Development of a fed batch fermentation strategy to produce bioethanol from non-detoxified hardwood spent sulphite liquor

by

Niel Bezuidenhout

Thesis presented in partial fulfilment

of the requirements for the Degree

of

MASTER OF ENGINEERING

(CHEMICAL ENGINEERING)

in the Faculty of Engineering

at Stellenbosch University

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

Supervisor

Prof. JF Görgens

Co-*Supervisor*

Dr Eugene van Rensburg

March 2021

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: *February 2021*

PLAGIARISM DECLARATION

1. Plagiarism is the use of ideas, material and other intellectual property of another's work and to present is as my own.
2. I agree that plagiarism is a punishable offence because it constitutes theft.
3. I also understand that direct translations are plagiarism.
4. Accordingly, all quotations and contributions from any source whatsoever (including the internet) have been cited fully. I understand that the reproduction of text without quotation marks (even when the source is cited) is plagiarism.
5. I declare that the work contained in this assignment, except where otherwise stated, is my original work and that I have not previously (in its entirety or in part) submitted it for grading in this module/assignment or another module/assignment.

Student number:

Initials and surname: N. Bezuidenhout

Signature: ……..

Date: February 2021

Abstract

Hardwood spent sulphite liquor (HW-SSL) from the pulp and paper industry is a potential substrate for bio-ethanol production, due to wide availability and high concentration of monomeric sugars (+100 g/L in concentrated streams). Current challenges such as low xylose consumption and high inhibitor concentrations need to be addressed to ensure the development of economically feasible fermentation processes. The work in this study aim to improve the maximum ethanol titre obtained in the fermentation of non-detoxified HW-SSL reported by previous studies (11.2-12.2 g/L), by implementing enhanced xylose capable *S. cerevisiae* strains along with a fed batch strategy to maximise *in situ* detoxification in the cultures. The study also prioritises the use of harsh industrially relevant conditions such as low cell densities (0.4-0.8 g/L dry weight), inexpensive nutrient sources as opposed to laboratory mediums, and utilising the SSL as-is, without any form of pretreatment or detoxification.

The characterisation of different industrial SSL streams indicated that the stream from the second last stage in a multi-effect evaporator is most suitable for fermentation. This high total dissolved solids (TDS) SSL stream contains a sufficiently high sugar concentration of 110 g/L to ensure that economically feasible ethanol titres of 40 g/L can be achieved. The utilisation of a stream that was concentrated in an evaporator is further beneficial as evaporation also served in removing some of the volatile inhibitors from the SSL by decreasing the furfural concentration from 0.6 g/L to 0.2 g/L and merely increasing the acetic acid concentration from 14 g/L to 17 g/L despite an increase of 200% in TDS content.

Preliminary strain screening experiments at different dilutions of the selected high TDS stream (20, 40, 60% (v/v) SSL) were conducted to compare novel strains and assess the influence of inhibitor concentrations on fermentation performance. CelluX™4, an advanced, xylose capable recombinant *Saccharomyces cerevisiae* strain, is the superior microorganism in 20-40% (v/v) SSL concentration fermentations as it displayed xylose capabilities, whereas *S. cerevisiae* strains TP1 and TFA7 proved less capable of xylose utilisation. The strain TFA7, however, proved to be more robust as it obtained an ethanol titre and volumetric productivity of 20-80% and 30% respectively, compared to the other two strains in high SSL concentrations of 60% (v/v). Although CelluX™4 possess the capability to consume xylose 50-100% faster than the other strains at 20% (v/v) SSL, this characteristic diminished at 60% (v/v) SSL concentrations due to the high inhibitor concentrations. It therefore appears that there was a trade-off between advanced xylose capabilities and inhibitor tolerance with CelluX™4

dominating the former and TFA7 the latter. CelluX™4 was selected for fed batch cultures in 5L bioreactor fermentations to assess the influence of substrate feeding.

This study is the first to utilise fed batch strategy for the fermentation of HW-SSL. Feeding cultures indicated that fed batch strategy allowed the fermentation of 65% (v/v) SSL media, with a TDS value of 35%, that is not otherwise fermentable in batch setup, as the batch culture obtained only an inconsistent ethanol titre of 1.6±1.6 g/L. When comparing different dilution rates, increasing the filling period from 6 days to 9 days resulted in a marginal ethanol titre increase from 9.5±0.4 g/L to 10.7±0.9 g/L at the expense of a 25% decrease in volumetric productivity. When doubling the inoculum size during the 6-day fermentation to 0.8 g/L, the ethanol titre and yield was increased by 35% and 20% respectively, reaching 12.7 g/L and 0.43 g/g, proving the advantage of a higher cell density. Allowing the double inoculum fed batch fermentation to continue in batch phase for another 4 days to partially consume residual sugars, further increased the ethanol titre to 15.5 g/L.

Despite the subjection of the yeast to harsher, industrially relevant conditions, the combination of utilising fed batch strategy and novel strain, CelluX™4 proved an efficient approach to mitigating inhibitor effects and increasing ethanol production (12.7-15.5 g/L) in non-detoxified HW-SSL compared to values reported in literature (11.2-12.2 g/L). Altering of the fermentation medium by detoxification or blending with other (lignocellulosic) glucose rich sources can be considered to further improve the ethanol production of the process.

Keywords: Hardwood spent sulphite liquor, Non-detoxified, *Saccharomyces cerevisiae*, Fed batch, Industrially relevant conditions

Opsomming

Hardhout gesuiterde sulfiet afloop (HW-SSL), afkomstig vanaf industriële verpulpingsprosesse, het die potensiaal om as substraat vir bio-etanol produksie gebruik te word, aangesien dit algeheel beskikbaar is en oor ‘n hoë monomeriese suikerkonsentrasie beskik (+100 g/L in gekonsentreerde strome). Dit is noodsaaklik om uitdagings soos lae xilose-verbruiksvermoë van giste en hoë inhibeerder konsentrasies in HW-SSL aan te spreek om die ontwikkeling van industrie-aanvaarbare fermentasie prosesse te verseker. Hierdie studie poog om die maksimum etanol konsentrasie wat behaal is in onbehandelde HW-SSL te verbeter, soos gerapporteer in vorige studies (11.2-12.2 g/L), deur gebruik te maak van moderne xilose-bekwame *S. cerevisiae* stamme en substraatvoermetodes om *in situ*-ontgifting deur bogenoemde giste te verbeter. Die studie lê ook klem op die toepassing van stramme industrie-aanvaarbare toestande soos lae gis konsentrasie (0.4-0.8 g/L droë massa), goedkoop voedingsstowwe in plaas van experimentele groeimediums, en die gebruik van HW-SSL sonder enige behandeling of ontgifting.

Die chemiese karaktisering van verskeie HW-SSL strome vanaf die papiermeul het aangedui dat die stroom wat afkomstig is van die tweede laaste verdampingsfase in ‘n multi-effek verdampingsisteem, die mees gespaste stroom vir fermentasie is. Hierdie HW-SSL stroom bevat ‘n hoë hoeveelheid totale opgeloste stowwe (TDS) (54%) sowel as totale suikerkonsentratie van 110 g/L, wat hoog genoeg is om ‘n ekonomies-aanvaarbare etanol konsentrasie van 40 g/L te verseker. Verdermeer het die verdamper ook daarin geslaag om van die inhibeerders uit hierdie gekonsentreerde stroom te verwyder aangesien die furfuraal konsentrasie vanaf 0.6 g/L na 0.2 g/L gedaal het en die asynsuur konsentrasie slegs vanaf 14 g/L na 17 g/L gestyg het, ten spite van ‘n 200% toename in TDS.

Basiese keuringseksperimente was uitgevoer in 20, 40 en 60% (v/v) SSL, om die funksionaliteit van die moderne gisstamme te vergelyk en die invloed van inhibeerderkonsentrasies op fermentasie vermoë te bepaal. Een van die verbeterde, gisstamme genaamd CelluX™4 is die mees dominante mikroorganisme in 20-40% SSL fermentasies aangesien dit uitstekende xilose-bekwaanheid ten toon gestel het, terwyl die twee *S. cerevisiae* stamme genaamd TP1 en TFA7, aansienlik minder instaat tot xilose-verbruiking was. TFA7 beskik wel oor die mees geharde natuur aangesien dit ‘n 20-80% en 30% verbetering in onderskeidelik etanol konsentrasie en volumetriese produktiwiteit teenoor die ander twee stamme in 60% (v/v) SSL behaal het. Alhoewel CelluX™4 xilose 50-100% vinniger as die ander stamme verbruik, word

hierdie vermoë negatief geaffekteer deur die hoë inhibeerderkonsentrasies by 60% (v/v). Dit wil dus voorkom asof daar ‘n kompromie tussen xilose-bekwaamheid en gehardheid in die modern giste bestaan waar CelluX™4 die eerste eienskap domineer en TFA7 die tweede. CelluX™4 was gekeur vir gebruik in die 5-L bioreaktor fermentasies om die invloed van substraatvoering op etanolproduksie to bepaal.

Hierdie studie is uniek in die sin dat voeringsmetodes voorheen nie op die fermentasie van HW-SSL toegepas is nie. Substraatvoering het dit moontlik gemaak om ‘n medium met 65% (v/v) HW-SSL en ‘n TDS van 35% te fermenteer, wat nie moontlik sonder voering was nie, aangesien slegs 1.6±1.6 g/L etanol geproduseer is wanneer al die HW-SSL vanaf die begin in die reaktor geplaas is. Deur die vullingstydperk van 6 dae na 9 dae te vermeerder is die etanol konsentrasie verhoog vanaf 9.5±0.4 g/L na 10.7±0.9 g/, teen die verlies van 25% in terme van volumetriese produktiwiteit. ‘n Verdubbeling in die bygevoegde gis konsentrasie, vanaf 0.4 g/L na 0.8 g/L, het die etanol konsentrasie en opbrengs vermeerder met onderskeidelik 35% en 20% vir die 6-dag fermentasie, wat dui op die voordeel van ‘n hoër gisbyvoeging. Deur die ekspirementstel met die hoë gisbyvoeging toe te laat om vir ‘n addisionele 4 dae te fermenteer, kon die etanol konsentrasie tot 15.5 g/L verhoog word.

Alhoewel die gis in hierdie studie aan meer stram, industrie-aanvaarbare toestande blootgestel is, was die gesamentlike effekte van die modern gisstam, CelluX™4, se fermentatsie vermoëns gekombineer met verskeie voerringsmetodes, genoeg om die negatiewe impak van die inhibeerders te verminder, en die etanol konsentrasie van nie-ontgiftigde HW-SSL soos gerapporteer in literatuur (11.2-12.2 g/L) te verbeter tot 12.7-15.5 g/L. Die etanol produksie kan verder verbeter word deur die fermentasie medium aan te pas met behulp van ontgiftiging of deur die vermenging van HW-SSL met ander lignosellulose bronne, wat ryk aan glukose is.

Sleutelwoorde: Hardhout gesuiterde sulfiet afloop, Nie-ontgiftigde, *Saccharomyces cerevisiae*, substraatvoering, industrie-aanvaarbare toestande

Acknowledgements

This research study was funded by the National Research Foundation (NRF). The author of the study would also like to thank the following people for their contributions:

Prof JF Görgens	For giving me the opportunity to practice research under his mentorship and motivation me to improve the quality of the study.
Dr Danie Diedericks **Dr Eugene van Rensburg**	For their valuable advice and support with all aspects of the study.
Dr Gerhardt Coetzee	For his patience and willingness to mentor, guide and train me in the correct laboratory procedures.
Ms Levine Simmers **Mr Jaco van Rooyen**	For completing the thankless task of analysing all the HPLC samples during the study. You exceeded expectations in all aspects.
Dr Bianca Brandt	For the supplication of microorganisms and advice on experimental approach.
Mr Alvin Petersen **Mr Heinrich Bock**	For all the logistical assistance during the project and selflessly sacrificing weekend time to allow me afterhours access.
Mr Thabo Gumede	For providing SSL from the Sappi Saiccor paper and pulp mill and hosting our visit to the premises.
Fellow students	Wellington Arthur and Eddie Barnard for laboratory assistance, especially during afterhours.
Loved ones	For your unconditional love, understanding and support in helping me achieve this goal.

Table of contents

Abstract iii

Opsomming v

Acknowledgements vii

Table of contents viii

List of Figures xi

List of Tables xiii

Acronyms and abbreviations xv

Glossary xvi

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: LITERATURE REVIEW 3

2.1 Feedstock 3

2.1.1 Origin and proposed integration in industrial process 3

2.1.2 Microbial inhibitors present in SSL 4

2.1.3 Wood pulping types 7

2.1.4 Challenges with xylose utilisation in HW-SSL and metabolic exhaustion 7

2.1.5 Effects of evaporator 10

2.1.6 pH adjustment 11

2.2 Past results 12

2.3 Microorganism 17

2.3.1 Desired phenotypes 17

2.3.2 Microorganism species 18

2.3.3 Recombinant *S. cerevisiae* strains 18

2.4 Improvement strategies 21

2.4.1 Pre-fermentation detoxification 22

2.4.2 Microbial *in situ* detoxification 22

2.4.3 Nutrient rich media 25
2.4.4 Microbe conditioning 25
2.4.5 Fed batch strategy 26
2.5 Gap in literature 28
2.6 Research questions 29
CHAPTER 3: RESEARCH DESIGN AND METHODOLOGY 31
3.1 Experimental approach 31
3.1.1 SSL stream selection - Characterisation 31
3.1.2 Shake flask batch fermentations 31
3.1.3 Fed batch concept 32
3.2 HPLC analysis 33
3.3 Feedstock characterisation 33
3.3.1 Source 33
3.3.2 Chemical characterisation 33
3.3.3 Total dissolved solids 33
3.4 Microbial strains 34
3.5 Inoculum preparation 34
3.6 Shake flask fermentations 35
3.6.1 Batch fermentations 35
3.6.2 Pulse fed batch fermentations 35
3.7 Large scale bioreactor experiments 36
3.8 Statistical analysis 36
3.9 Fermentation performance analysis 37
CHAPTER 4: RESULTS AND DISCUSSION 38
4.1 SSL characterisation – the feedstocks 38
4.1.1 Sugar content 40
4.1.2 Inhibitor content 42

4.2 Baseline Fermentation - Synthetic media....44
4.3 Batch fermentation in SSL....47
4.4 Sensitivity of xylose capability in *S. cerevisiae* strains in HW-SSL fermentations .52
4.5 Fed batch fermentations....54
4.5.1 Shake flask pulse-fed fermentations....54
4.5.2 Fed batch fermentations with continuous feeding in a 5L bioreactor....58
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS....64
5.1 Conclusions....64
5.2 Recommendations....65
REFERENCES....67
APPENDIX....78
Fermentation of low TDS SSL (Mg-4)....78
Raw data....81

List of Figures

Figure 1: The integration of a fermentation plant into a pulping process adapted from Petersen et al. (2014) 4
Figure 2: The diminishing of xylose consumption ability in recombinant *S. cerevisiae* strains due to an increase in acetic acid concentration. The data was adapted from the following studies: SXA-R2P-E (Ko, Um and Lee, 2016); NAPX37 (Li et al., 2017); XUSE, XUSAE57 (Ko et al., 2020). 9
Figure 3: The development of robust, xylose capable recombinant *S. cerevisiae* strains for ethanol production in lignocellulosic materials 19
Figure 4: Shake flask fermentations under microaerophilic conditions, ensured by rubber stoppers and s-shaped airlock, carried out in an incubator 31
Figure 5: Fed batch fermentations carried out in a bioreactor. 32
Figure 6: Relationship between TDS content and sugar concentrations across different stages in the multi-effect evaporator as represented in Table 8 40
Figure 7: Total sugar contents of different SSL streams. The different required marks signify assumed product yield values 41
Figure 8: Inhibitor concentrations of streams Mg-5 to Mg-1A in the multistage evaporator. . 42
Figure 9: Fermentation profiles of CelluX™4 (A) and TFA7 (B) in synthetic medium. Horizontal arrows indicate the different sugar phases of the fermentations 44
Figure 10: Ethanol titre (A), Productivity (B) and xylose consumption rate (C) during different sugar phases in the fermentation of synthetic medium 45
Figure 11: Fermentation profiles of different yeast strains at 20% SSL (A, B), 40% SSL (C, D), and 60% SSL (E, F) 48
Figure 12: Acetic acid detoxification in 20% and 40% SSL fermentations 51
Figure 13: Fermentation profiles in batch cultures of 60% SSL (A) and 60% SSL supplemented with 20 g/L glucose (B) by CelluX™4 53
Figure 14: Fermentation profiles of 60% SSL for batch fermentation (A; D), fed batch configuration for two-pulse (B; E) and four-pulse (C; F). The arrows represent the times at which SSL pulses were administered. 55
Figure 15: Xylose consumption rate (A), productivity (B), ethanol yield (C) and titre increases (D) for different phases in the pulse fed batch fermentation 57
Figure 16: Change in SSL loading (solid lines) and volumetric feeding rate (dotted lines) over time in fed batch bioreactor fermentations compared to simple batch cultures 58

Figure 17: Ethanol production profiles over time of batch and fed batch fermentations in 5-L bioreactors 59
Figure 18: Sugar consumption (A) and acetic acid consumption (B) in bioreactor cultures ... 60
Figure 19: Fermentation profiles of Mg-4 SSL in batch culture under different oxygen conditions facilitated by rubber and cotton stoppers respectively 79
Figure 20: Inhibitor degradation during the fermentation of Mg-4 SSL facilitated by rubber and cotton stoppers respectively 80

List of Tables

Table 1: Comparison of thin and thick HW-SSL streams in literature 10
Table 2: Ethanol production from non-detoxified HW SSL 13
Table 3: Most successful studies for ethanol production from non-detoxified HW SSL 15
Table 4: Compositions of the substrates fermented using the advanced yeast strains 20
Table 5: Summary of lignocellulosic improvement strategies and associated compromises on industrial fermentation goals 21
Table 6: The combination of process steps in lignocellulosic liquid fermentations by means of recombinant microbial improvements 23
Table 7: Fermentation parameters calculations 37
Table 8: Composition of SSL streams from the CaO cooking line and MgO evaporation line with Mg-5 to Mg-out in increasing thickness. 39
Table 9: Kinetic parameters obtained in fermentation of synthetic medium 44
Table 10: Ethanol production parameters obtained in the fermentation of 20%, 40% and 60% (v/v) SSL fermentation by strains CelluX™4, TP1 and TFA7 47
Table 11: Different pulse feeding schemes for fed batch fermentation of SSL 54
Table 12: Ethanol production parameters obtained in 60% SSL batch and fed batch fermentations 55
Table 13: Ethanol production parameters in 5-L bioreactor batch and fed batch fermentations 59
Table 14: Ethanol production from non-detoxified HW SSL 62
Table 15: Fermentation parameters of Mg-4 SSL in batch culture under different oxygen conditions 79
Table 16: CelluX^{TM}4 culture in synthetic media 81
Table 17: TFA7 culture in synthetic media 81
Table 18: CelluX^{TM}4 culture in 20% SSL media 82
Table 19: TP1 culture in 20% SSL media 82
Table 20: TFA7 culture in 20% SSL media 83
Table 21: CelluX^{TM}4 culture in 40% SSL media 84
Table 22: TP1 culture in 40% SSL media 85
Table 23: TFA7 culture in 40% SSL media 86
Table 24: CelluX^{TM}4 culture in 60% SSL media 87
Table 25: TP1 culture in 60% SSL media 87

Table 26: TFA7 culture in 60% SSL media....88
Table 27: CelluX^{TM}4 two-pulse shake flask fed batch culture....88
Table 28: CelluX^{TM}4 four-pulse shake flask fed batch culture....89
Table 29: TFA7 two-pulse shake flask fed batch culture....90
Table 30: TFA7 four-pulse shake flask fed batch culture....91
Table 31: Reactor 6-day fed batch culture....92
Table 32: Reactor 9-day fed batch culture....92
Table 33: Reactor 6-day fed batch double inoculum culture....93
Table 34: Reactor batch culture....93

Acronyms and abbreviations

CSL	Corn steep liquor
EtOH	Ethanol
HMF	5-hydroxymethylfurfural
HPLC	High performance liquid chromatography
HW-SSL	Hardwood SSL
R/O	Reverse osmosis
SSL	Spent sulphite liquor
SW-SSL	Softwood SSL
TDS	Total dissolved solids
YE	Yeast extract
YPD	Yeast extract, peptone, glucose
YPX	Yeast extract, peptone, xylose

Glossary

Coculture	Fermentations where two or more microorganism strains or species are inoculated into the media, as both serve separate functions
Co-fermentation	Fermentations where both glucose and xylose are used as carbon sources
Conditioning	The process in which microorganisms are gradually acclimated to an inhibitor rich media by growing it in a diluted form of the media.
Detoxification	The removal of inhibitors from the fermentation media either before or during the fermentation (see *in situ* detoxification and preculture detoxification).
Fed batch	The feeding of additional media into the system during fermentation, without removing any liquid.
Genetic engineering	The practice of emphasising desirable traits by means of genetic manipulation to improve fermentation performance of microorganisms.
***In situ* detoxification**	The removal of inhibitors during the fermentation itself, rather than detoxification before the fermentation (See preculture detoxification).
Inhibitors	Chemical compounds present in fermentation media that restricts the microbial performance by affecting cell vitality and metabolism.
Preculture detoxification	The removal of inhibitors from the fermentation media in a separate step before fermentation.
Recombinant microorganism	A microorganism comprised of genetic fragments from different sources combined.
Saccharomyces cerevisiae	The yeast species more generally known as ‘baker’s yeast’, which is used for most industrial ethanol production fermentations.
Spent sulphite liquor	A liquid waste stream produced in acidic sulphite pulping mills after the valuable cellulose has been washed and removed for further pulping.
Microbial strain	A genetic subgroup within a species.
Total dissolved solids content	The mass percentage contributed to a solution by the dissolved solids such as salts or sugars

CHAPTER 1: INTRODUCTION

Bio-ethanol production is essential to the process of developing more sustainable fuels for road transport and aviation, since it can be used in dilution with existing petroleum derivatives as well as in its pure form (Hamelinck et al., 2005). Ethanol also finds application as an industrial solvent, platform chemical and disinfectant. One opportunity for industrial ethanol production is the effective integration of an ethanol fermentation plant into an existing paper and pulp mill, by using waste streams as substrate. Hardwood spent sulphite liquor (HW-SSL; hereinafter just SSL) generated from acidic sulphite pulping processes has been identified as an attractive substrate, due to its relatively high concentrations of fermentable monomeric sugars, ranging between 2-20 g/L glucose and 20-120 g/L xylose (Harner et al., 2014). SSL is also advantageous due to its high availability and low cost (Holderby and Moggio, 1960; Novy et al., 2013). Although SSL produced from softwood (SW-SSL) pulping has already been widely implemented as fermentation substrate (Lawford and Rousseau, 1993; Pereira et al., 2013), the use of HW-SSL is still in the development phase.

HW-SSL fermentations are more challenging due to the inefficient metabolising of xylose by most yeast strains, further impeded by the high concentrations of inhibitory compounds in SSL (Deparis et al., 2017; Helle et al., 2004; Moysés et al., 2016). The ability of *S. cerevisiae* strains to utilise xylose is much more sensitive to the inhibitors present in SSL than that of glucose, making HW-SSL a particularly challenging fermentation medium (Deparis et al., 2017; Li et al., 2017; Moysés et al., 2016).

The SSL residue stream is often used in energy recovery by incineration, after a multistage evaporator rids the stream of its excess water. This allows for the generation of streams of different sugar concentration (30-130 g/L), since this evaporator concentrates the medium in multiple stages prior to combustion, each of which may be considered as feedstock for fermentation (Chipeta et al., 2005; Helle et al., 2004; Marques et al., 2009; Rueda et al., 2015). However, SSL with a sugar concentration of more than 100 g/L has a high dissolved solids content of ±60% mass (Brandt, 2019; Marques et al., 2009; Rueda et al., 2015), which puts the microorganisms under excessive stress (Helle et al., 2008; Taherzadeh and Karimi, 2011; Xavier et al., 2010). Dilute streams (also known as thin SSL) might have the advantage of lower solids loading (10-20 %) but comes at the expense of a lower sugar concentration (25-35 g/L) (Chipeta et al., 2005; Helle et al., 2004; Rueda et al., 2015). This will in turn lead to

lower final ethanol concentrations, which will cause distillation cost to drive the process towards infeasibility (Holderby and Moggio, 1960; Petersen et al., 2014).

One way to make concentrated SSL streams more attractive for ethanol production is through detoxification prior to fermentation, which in turn increases ethanol yield, productivity and titre (Chandel et al., 2013; Helle et al., 2008; Nigam, 2001). Amongst others these methods include alkali treatment, adsorption with activated carbon or ionic exchange resins, evaporation, stripping, liquid-liquid-extraction, ultra-filtration and the addition of chemical reducing agents and enzymes (Alriksson et al., 2011; Jonsson et al., 2013; Llano et al., 2015, 2017). Detoxification methods will however often require a separate process step and/or lead to an increase in process and capital cost, since extra units, chemicals and/or enzymes are required (Holderby and Moggio, 1960; Rivard et al., 1996). An ideal fermentation process would therefore be to use non-detoxified SSL to avoid the additional detoxification cost associated with the integrated ethanol plant.

It has been shown that some genetically engineered yeast strains are resistant to inhibitors found in hydrolysates produced from lignocelluloses, such as SSL (Heer and Sauer, 2008; Helle et al., 2004). Some of these advanced strains can remove certain inhibitor compounds by means of *in situ* detoxification, while producing ethanol, thus eliminating the need for pre-fermentation detoxification of the HW-SSL (Brandt, 2019; Kim, 2018; Taherzadeh and Karimi, 2011). The detoxification ability of the yeast strains can be further improved by means of hardening strains towards inhibitors, overexpressing genes known to increase robustness and using a larger inoculum (Jonsson et al., 2013; Ko, Um, Woo, et al., 2016; Pereira et al., 2015; Xavier et al., 2010). While inhibitor tolerance is a critical criterium for microbial strain selection, it is equally important to xylose utilisation capabilities. Both robustness and xylose affinity are required phenotypes in yeast strains to maximise ethanol production.

Another inhibitor mitigating strategy is the usage of fed batch cultivation as this allows strains to maximise performance in a less toxic environment and provides a period for cells to adapt to high inhibitor concentrations (Modig et al., 2008; Nilsson et al., 2001; Zhang et al., 2014). The hypothesis is that a more concentrated stream will be fermentable by fed batch strategy than by batch, resulting in a higher final ethanol concentration (Taherzadeh and Karimi, 2011). Since there might be a productivity-yield trade-off (Han et al., 2017), it is necessary to experimentally investigate different feeding profiles, to provide process data for future techno-economic studies, to determine preferred operational conditions.

CHAPTER 2: LITERATURE REVIEW

2.1 Feedstock Origin and proposed integration in industrial process

Spent sulphite liquor (SSL) is a by-product of the acidic sulphite pulping process as can be seen from Figure 1. The chemical digestion of woodchips solubilises/hydrolyses the hemicellulose and lignin components, leaving the insoluble, valuable cellulose, which is subsequently sent for further refining and bleaching. SSL is the remaining liquid residue stream generated after the cellulose solids have been washed, containing the chemicals from digestion and the dissolved hemicellulose- and lignin fractions of the wood. Many pulping mills recover the residual chemicals by incinerating the SSL in a furnace, which in turn also provides additional energy to the system. The SSL is concentrated through a multistage evaporation process to produce a combustible final product.

The multi-effect evaporator provides many streams of different total dissolved solids (TDS) content, presenting the opportunity to select a stream with a sufficient sugar content (Figure 1). The criteria and selection of an SSL stream for use in a fermentation process will be discussed in more detail in Section 2.1.5. After the selected stream is fermented, the ethanol is recovered by means of distillation. Petersen et al. (2014) stated that the distillation residue can be returned to an evaporation stage with a suitable dissolved solids content, for further concentration to ensure efficient incineration.

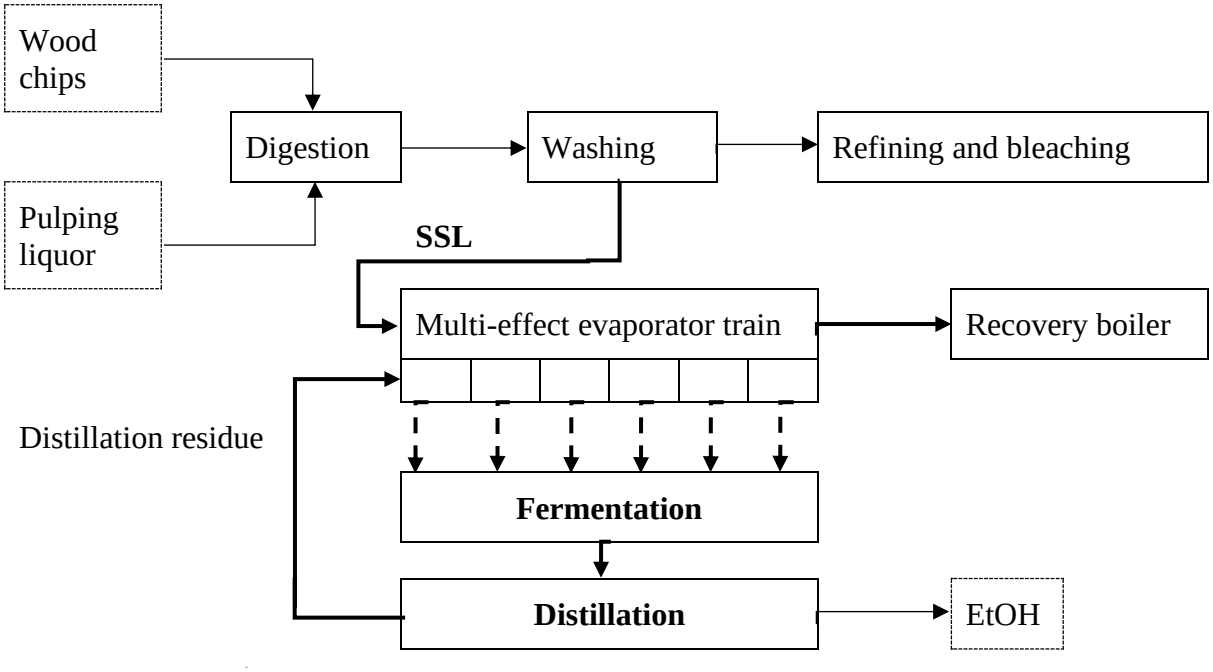

Figure 1: The integration of a fermentation plant into a pulping process adapted from Petersen et al. (2014)

2.1.2 Microbial inhibitors present in SSL

Apart from the monomeric and oligomeric sugars, a wide range of by-products are also released during the digestion of the wood chips. Many of these are considered microbial inhibitors, as they upset the metabolic processes of the microorganisms during the fermentation of SSL (Helle et al., 2008; Johansson et al., 2011). The most common of these are furan aldehydes, weak organic acids and phenolic compounds (Jonsson et al., 2013; Kim, 2018; Taherzadeh and Karimi, 2011).

2.1.2.1 Furan aldehydes

The dehydration of pentose and hexose sugars leads to the formation of furfural and 5-hydroxymethylfurfural (HMF) respectively (Kim, 2018; Taherzadeh and Karimi, 2011). These furan derivatives can impede the activity of regulating enzymes and damage organelle membranes within microorganism cells (Taherzadeh and Karimi, 2011). The outcome of such damage to the cell physiology is a decrease in cell growth, sugar uptake and ethanol production (Chandel et al., 2012; Jonsson et al., 2013). Both furfural and HMF can however be reduced to less toxic compounds by the fermenting organisms themselves, if concentrations are sufficiently low; usually below 1 g/L but values are dependent on the yeast strains and presence of other inhibitors (Pereira et al., 2013; Taherzadeh and Karimi, 2011). Furfural is a more harmful inhibitor than HMF (Kim, 2018), and is also more prominent in HW-SSL, as hexose sugars degrade less readily than pentose sugars (Chandel et al., 2012). The concentration of

furfural in HW-SSL can vary from 0-2 g/L and is dependent of wood composition and process conditions (Table 1) and can therefore either be a threat or harmless.

2.1.2.2 Aliphatic acids

The most common weak acids produced in the pretreatment of lignocellulosic material is acetic acid, formic acid and levulinic acid (Jonsson et al., 2013; Taherzadeh and Karimi, 2011). Acetic acid is produced from the deacetylation of hemicellulose fractions, while formic acid and levulinic acid are the degradation products of the abovementioned furan aldehydes (Jonsson et al., 2013; Taherzadeh and Karimi, 2011). Although acetic acid is the least toxic of the three acids , it is present at high concentrations of 5-12 g/L in HW-SSL (Chipeta et al., 2005; Marques et al., 2009; Rueda et al., 2015) compared to trace amounts of the other acids, thus proving to pose the largest threat to fermentations of the three acid compounds (Ko et al., 2020; Li et al., 2017; Taherzadeh and Karimi, 2011). Organic acids are most inhibiting when present in the undissociated form, as these molecules enter the microorganism through the cell membrane and dissociate in the cytoplasm due to the higher pH 7 compared to pH 5 of the medium (Jonsson et al., 2013; Kim, 2018). Dissociation inside the cells lead to acidification of the cytoplasm, which in turn leads to the impedance of sugar and ion transport and thus decreasing cell growth and ethanol production (Jonsson et al., 2013; Kim, 2018). Microorganisms can restore the intercellular pH to neutral by using ATP to pump protons from the dissociated acids out of the cell, which can incidentally increase the ethanol yield by limiting cell growth at low acetic acid concentrations (< 2 g/L) (Jonsson et al., 2013; Ko, Um and Lee, 2016). At high acetic acid concentrations however, intercellular pH is not easily regulated due to a lack of energy supply in the form of ATP, which in extreme cases can lead to cell death (Jonsson et al., 2013; Taherzadeh and Karimi, 2011).

2.1.2.3 Phenolic compounds

Phenolic compounds are formed mainly from the degradation of lignin but can also be formed from the breakdown other aromatic chemical species such as sugars (Jonsson et al., 2013; Taherzadeh and Karimi, 2011). Compounds such as vanillin, vanillic acid, syringaldehyde, syringic acid 4-hydroxybenzoic acid, coniferyl aldehyde and ferulic acid has been studied as inhibitors of metabolic processes (Jonsson et al., 2013; Kim, 2018; Taherzadeh and Karimi, 2011). Although these chemical species are known to exist in much lower concentrations than other inhibitors, they are much more toxic due to the ability to diffuse across the cell membranes (Kim, 2018; Taherzadeh and Karimi, 2011). Dependent on functional groups, the lethal concentration can be 1-2 g/L although concentrations as low as 0.1 g/L can lead to

inhibition (Chandel et al., 2013; Li et al., 2017). The mechanism of inhibition differs depending on the functional groups of the compounds, but it is widely accepted that the phenolic compounds negatively affect the functionality and integrity of cell membranes (Chandel et al., 2012; Jonsson et al., 2013). The decay of membrane structure in turn leads to a less efficient selective permeability, leading to less effective sugar transport and possible nutrient losses (Chandel et al., 2012; Taherzadeh and Karimi, 2011).

2.1.2.4 Synergistic effects

Fermentation media in which multiple inhibitor types are present impede the metabolic processes in microorganisms more than the sum of the individual inhibitor effects (Jonsson et al., 2013; Kim, 2018). Therefore, there exist synergistic effects between different chemical species, which cause the lethal concentration of an inhibitor to be lower in a medium with other inhibitors than it would be in a medium where the compound was alone (Klinke et al., 2003; Li et al., 2017; Palmqvist et al., 1999) The combinations of metabolic obstruction mechanisms result in cells losing viability and will in severe cases lead to cell death (Liu et al., 2019; Taherzadeh and Karimi, 2011).

2.1.2.5 High dissolved solids content

The dissolved solids fraction in SSL consists of a mixture of monomeric sugars, oligomers, inorganic salts and degraded lignin derivatives. The total dissolved solids (TDS) content is the mass fraction dissolved solids in the SSL and ranges from 10-60% (Table 1) depending on whether the liquid has been concentrated by means of evaporation. SSL with a TDS value of 30% are considered to be inhibiting to fermenting organisms, due to the high osmotic stress caused by dissolved solids (Helle et al., 2004, 2008; Petersen et al., 2014). SSL with TDS values above 30% are also mostly formed by means of concentration in the multistage evaporator as discussed in Section 2.1.1. The evaporation process leads to an increase in concentration of the involatile compounds such as inorganic salts, lignosulphonates and phenolic compounds, which can all further inhibit the functionality of microorganisms (Jonsson et al., 2013; Taherzadeh and Karimi, 2011).

2.1.3 Wood pulping types

There are many factors that determine the chemical composition of SSL streams from pulping. The type of digestion process plays a large role in the mechanism of removing lignin and hemicellulose from cellulose in the wood chips (Pereira et al., 2013), which will in turn also influence the composition of the spent liquor. The extreme chemical and physical treatment undergone by the wood, renders SSL a highly inhibitory feedstock (Almeida et al., 2007; Delgenes et al., 1996; Schimz, 1980), as discussed in greater detail in Section 2.1.2.

One of the largest influences on SSL composition is the type of wood used as feedstock for the pulping process (Pereira et al., 2013). There are notable differences between the SSL produced by softwood (SW-SSL) and that of hardwood (HW-SSL). Industrial scale fermentation of SW-SSL has already been widely implemented in some areas in the northern hemisphere (Pereira et al., 2013) as 74-76% of the available sugars are hexose (glucose and mannose) rendering it ideal for fermentation by *S. cerevisiae* (Helle et al., 2004; Lawford and Rousseau, 1993). HW-SSL on the other hand is yet to be utilised on a large scale as only 11-30% of sugars are hexoses, and the remaining pentose sugars (predominantly xylose) is not naturally fermentable by *S. cerevisiae* (Chipeta et al., 2005; Lawford and Rousseau, 1993; Rueda et al., 2015). Subsequently HW-SSL also has a higher weak acid and furfural concentration than SW-SSL due to the breaking down of glucuronoxylan (Chandel et al., 2012; Jonsson et al., 2013; Marques et al., 2009). The inability of *S. cerevisiae* as the preferred industrial fermentation microbe, to metabolise xylose in the presence of high concentrations of inhibitors is one of the major challenges for effective fermentation of HW-SSL on an industrial scale. The use of advanced yeasts and well-developed feeding schemes could make the fermentation of HW-SSL possible.

2.1.4 Challenges with xylose utilisation in HW-SSL and metabolic exhaustion

S. cerevisiae, which is the preferred microorganism for industrial bioethanol production, is not naturally capable of fermenting xylose, as it lacks efficient xylose transporters and metabolic pathways, and consumption can lead to an upset of its cell homeostasis (Bergdahl et al., 2012; Cunha, Romaní, et al., 2019). Much work has been done to modify this species for xylose consumption by the insertion of genes from other microorganisms. The two most prominent xylose utilising pathways are the xylose reductase-xylitol dehydrogenase (XR/XDH) and xylose isomerase (XI), which are naturally present in some fungal and bacterial species,

respectively (Moysés et al., 2016). The XR/XDH pathway can lead to redox imbalances in oxygen limiting conditions, whereas the XI pathway can eliminate this limitation (Zhang et al., 2016). The XI pathway tends to lead to higher ethanol yields whereas the XR-XDH pathway can achieve a higher xylose utilisation rate and therefore productivity (Cunha, Soares, et al., 2019).

Xylose utilisation in lignocellulosic materials is further inhibited by means of glucose repression since both xylose and glucose compete for the same transporters into the yeast cells. In many cases the transporters have a higher affinity to glucose than xylose, resulting in stifled xylose uptake at high glucose concentrations (Avanthi et al., 2017). There are however indications that catabolite repression does not occur at glucose concentrations between 0-2 g/L and xylose uptake is even improved, as these small amounts of glucose appears to activate receptors to enhance xylose transport into the yeast cells (Lane et al., 2018; Meinander and Hahn-Hägerdal, 1997; Souto-Maior et al., 2009).

Even recombinant *S. cerevisiae* strains specifically engineered with xylose utilising capabilities are limited in maintaining this ability, as the low xylose consumption rate can cause cells to deplete metabolites such as energy sources and cofactors in the cell at a faster rate than it can be produced (Bergdahl et al., 2012; Matsushika et al., 2014). The lack of these metabolites results in restricted growth and cell maintenance which leads to a decrease in cell viability and ultimately carbon starvation (Matsushika et al., 2013; Wei et al., 2018).

Xylose fermentation is furthermore challenged by the toxic nature of lignocellulosic material such as SSL as microbial inhibitors put more stress on the microorganisms, thus consuming internal metabolites even faster and leading to metabolic exhaustion (Moysés et al., 2016; Wang et al., 2014). The inhibition of glucose fermentation is largely coupled with impediment of culture growth, whereas xylose utilisation is more directly affected due to microorganisms losing the ability to assimilate this sugar rendering it more sensitive to inhibition than glucose (Deparis et al., 2017; Li et al., 2017). Xylose capable *S. cerevisiae* strains are inherently less robust, as the insertion of xylose fermenting ability into the genome can compromise existing inhibitor tolerance phenotypes (Bellissimi et al., 2009; Cunha, Romaní, et al., 2019). Brandt (2019) also found that genetic modifications to promote robustness in glucose fermentation did not always relate to better inhibitor tolerance of the xylose metabolism. This is a major additional hurdle for the fermentation of HW-SSL, as xylose contributes 70-85% of the available sugar content (Chipeta et al., 2005; Lawford and Rousseau, 1993; Rueda et al., 2015).

In order to illustrate the detrimental effect of inhibitors on xylose consumption Figure 2 illustrates the sensitivity of different xylose capable recombinant *S. cerevisiae* strains to acetic acid, as acetic acid is one of the major inhibitors in lignocellulosic feedstocks (Ko et al., 2020; Taherzadeh and Karimi, 2011). An acetic acid concentration of higher than 5-6 g/L acetic acid is inhibitory to fermentation (Jönsson and Martín, 2016; Kim, 2018). This can however be lower for xylose fermentations especially in the presence of other inhibitor species (Li et al., 2017). All fermentations were carried out in 20-40 g/L synthetic xylose cocktail at pH 5 and initial inoculum density of OD_{600} 1-2 with the exception of (Li et al., 2017) which also contained 60 g/L of glucose.

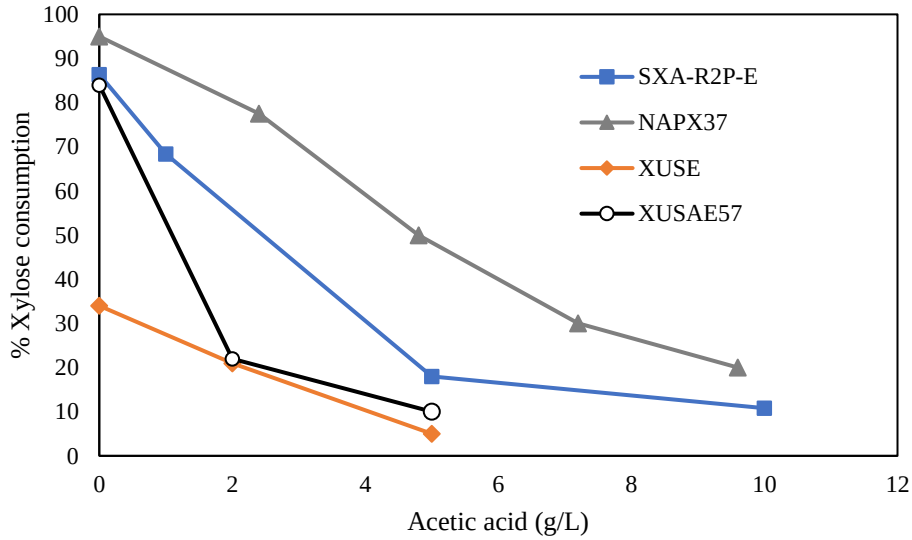

Figure 2: The diminishing of xylose consumption ability in recombinant *S. cerevisiae* strains due to an increase in acetic acid concentration. The data was adapted from the following studies: SXA-R2P-E (Ko, Um and Lee, 2016); NAPX37 (Li et al., 2017); XUSE, XUSAE57 (Ko et al., 2020).

An increase in acetic acid concentration leads to a gradual decrease in the ability of strains to utilise xylose. Except for the strain NAPX37 (Li et al., 2017), all the studies showed a complete impedance of xylose capability at 5 g/L acetic acid. This is in accordance with the heuristics stipulated earlier and can therefore be considered a lethal concentration for xylose fermentations.

2.1.5 Effects of evaporator

SSL is often available at multiple dissolved solids concentrations since the stream is concentrated in a multi-effect evaporator. In the process the sugar concentration is also increased. Industrial scale ethanol production will only be economically feasible if SSL is concentrated by means of evaporation before fermentation, as these 'thick' stream contain more than 100 g/L sugars as opposed to the 25-35 g/L of 'thin' streams before evaporation (Brandt, 2019; Helle et al., 2004; Marques et al., 2009; Rueda et al., 2015). The high sugar concentration of thick SSL is required to obtain ethanol concentrations of 40 g/L, which is the minimum titre limit to justify distillation costs (Pereira et al., 2013; Petersen et al., 2014). However, increasing the dissolved content of the fermentation medium, could have adverse effects on microorganism metabolism, as it leads to osmotic stresses in cells (Helle et al., 2008; Taherzadeh and Karimi, 2011; Xavier et al., 2010). It is therefore necessary to find an SSL concentration with high sugar content while keeping the inhibition at acceptable levels.

Xylose can also be dehydrated into furfural derivatives in the SSL concentrations steps, actually leading to a decrease in sugar concentration at very low concentrations (Marques et al., 2009; Novy et al., 2013). It is necessary to determine whether this is the case, since both a decrease in xylose concentration and increase in furfural concentration is unfavourable. The extent of sugar degradation is however expected to be low, meaning that sugar concentration will generally increase as water is evaporated. In order to provide insight into the relationship between inhibitor and sugar concentrations, Table 1 summarises the literature on the comparison of thick and thin SSL composition.

Table 1: Comparison of thin and thick HW-SSL streams in literature

	(Marques et al., 2009)		**(Chipeta et al., 2005)**		**(Rueda et al., 2015)**		**(Petersen et al., 2014)**	(Brandt, 2019)
SSL type	**Thin**	**Thick**	**Thin**	**Thick**	**Thin**	**Thick**	**Thin**	**Thick**
Glucose (g/L)	3.54	10.36	2.2	10.9	2.35	19.21	1.38	14.7
Xylose (g/L)	24.78	81.4	23.6	119.0	25.01	138.2	18.25	92.7
Acetic acid (g/L)	9.44	4.44	10.3	12.6	6.92	5.03	4.26	15.1
Formic acid (g/L)	-	-	-	-	-	-	0.81	0.56
Furfural (g/L)	2.36	traces	-	-	0.17	0.12	0.29	2.08
HMF (g/L)	-	-	-	-	0.03	0.04	0.0045	0.21
% solids	12.8.	56.8-	-	-	10.72	58.5	10.95	60
Solids (g/L)	151	841	145	753	-	-	-	-
pH	2.9	3.7	2.7	3.3	1.8	2.35	-	-

Acetic acid is the inhibitor with the highest concentration (Table 1) and will therefore be used as the parameter to calculate sugar/inhibitor ratios. In the cases of Marques et al. (2009); Rueda et al. (2015) 90 % of the acetic acid was removed, causing the sugar to acetic acid ratio to increase from 3-4 g/g to 20-30 g/g, indicating that evaporation favoured the fermentation process. In both these cases the acetic acid concentration was also decreased from 7-9 g/L to 4-5 g/L, as the acetic acid was evaporated along with the water, meaning that the thick SSL will likely be less toxic than the thin SSL.

In the cases of Brandt (2019); Chipeta et al. (2005); Petersen et al. (2014), 35-75% of acetic acid was removed. However, the sugar to acetic acid ratio only increased from 2.5-4.6 g/g to 7.1-10 g/g, indicating a much lower improvement in acetic acid removal compared to the above cases. These two cases also showed an increase in acetic acid concentration from 10.3 g/L to 12.6 g/L and 4.3 g/L to 15.1 g/L respectively, rendering the thick SSL more toxic than the think SSL.

The differences in inhibitor removal observations are likely connected to the different evaporator schemes and associated operating pressures. The comparison of Petersen et al. (2014) and Brandt (2019) from the same mill (Sappi Saiccor, Umkomaas, South Africa) shows a clear increase in acetic acid concentration in the thick SSL stream, implying that the thick stream might be more inhibitory than the thin stream. It is worth investigating the possibility of a trade-off between sugars and inhibitors of the different streams, especially since this mill will also be supplying the feedstock for this study.

2.1.6 pH adjustment

Untreated SSL is generally acidic with a pH in the range of 2-4. In order to mitigate the inhibition effects of weak organic acids, the pH of the substrate needs to be increased to 5-5.5 to facilitate dissociation of these compounds (Pampulha and Loureiro-Dias, 1989). It is essential to maintain the pH at this set-point, by means of buffer or feedback control, since yeasts operate optimally at pH 5-5.5 and decreases in pH during fermentation can lead to productivity decreases (Coote and Kirsop, 1976; Helle et al., 2004; Novy et al., 2013)

2.2 Past results

High ethanol yield and productivity can be obtained in the fermentation of medium that has been detoxified by implementing the methods which will be discussed in Section 2.4.1. The focus of this study, however, is the maximisation of ethanol production from non-detoxified HW SSL. Table 2 displays the best performing microorganisms and processing conditions in studies conducted on the fermentation of non-detoxified HW-SSL, as reviewed by the authors of the current study as well as the studies by Pereira et al. (2013) and Branco et al. (2019).

Most of the studies obtained an ethanol titre within the range 4-8 g/L except three studies which produced more than 10 g/L ethanol, thus emphasising the difficulty of fermenting non-detoxified HW-SSL. The low ethanol concentrations can be attributed to a combination of high inhibition, low total sugar and low sugar conversion. Regrettably, most studies only reported acetic acid concentration as an indication of inhibition. The acetic acid concentration for the fermentations were in the range of 5-10 g/L, which is considered detrimental for xylose utilisation by *S. cerevisiae* as discussed in Section 2.1.4.

Another factor which influences the low ethanol titre, is the low concentration of fermentable sugars, which stems from the low TDS content of the SSL. Most of the studies used SSL with a TDS value in the range 16-22%, with a total sugar content of 30-45 g/L. In studies where a higher sugar concentration is available due to a higher TDS value, the performance is limited by either lower ethanol yields of 0.2-0.3 g/g and/or incomplete xylose utilisation.

The focus of this study is to maximise the ethanol titre in non-detoxified HW-SSL fermentations. The aim is to reach 12 g/L of ethanol, since this is the highest concentration thus far obtained in literature to the knowledge of the author. The studies by Helle et al. (2004), Henriques et al. (2018) and Lawford and Rousseau, (1993) are the only studies where more than 10 g/L ethanol was produced from non-detoxified HW-SSL as also reviewed by (Pereira et al., 2013) and (Branco et al., 2019). It is unknown why these studies specifically produced better results than the other fermentations found in literature, since comparison between different organisms and media sources are often difficult. There are however key process conditions which potentially contributed to the high ethanol titre and yield obtained in the three studies which will likely not be implementable under industrial conditions. The process conditions of the best performing fermentations are presented in Table 3.

Table 2: Ethanol production from non-detoxified HW SSL

Paper mill	% TDS[1]	% SSL[2]	TSC[3] (g/L)	Acetic acid (g/L)	Time (h)	Species (strain)	EtOH (g/L)	Productivity (g/L·h)	$Y_{P/S}$ (g/g)	Xylose consumption (%)	Reference
						S. cerevisiae					
Sappi, Saiccor	36	60	50.1	5	72	CelluX™1	6.76	0.094	-	11.8	(Brandt, 2019)
			50.1			TFA40	7.02	0.097	-	11.2	
			50.1			TP50	7.3	0.101	-	11.4	
Sappi, Saiccor	48	80	70.6	7	72	CelluX™1	5.89	0.082	-	6.5	(Brandt, 2019)
			70.6			TFA40	6.81	0.095	-	5.6	
			70.6			TP50	5.99	0.083	-	6.6	
Tembec, Témiscaming	**20**	**100**	**36.7**	**-**	**120**	**259ST**	**12**	**0.1**	**0.42**	**65**	**(Helle et al., 2004)**
Tembec, Témiscaming	16	70	15	-	NS	259ST	6-7	-	-	100	(Helle et al., 2008)
Tembec Inc	**NS[4]**	100	29.1	10	72	R57	2.8	0.039	0.4	NS	(Pinel et al., 2011)

[1] TDS – Total dissolved solids mass percentage
[2] Applicable when SSL is diluted with water to lower inhibitor concentrations. Corresponding sugar and inhibitor concentrations are the final amounts in the fermentation.
[3] TSC – Total sugar concentration
[4] When compared to SSL in other studies from the same paper mill and with a similar sugar content, the TDS is likely 20-22%

						S. stipitis					
Caima-Indústria de Celulose SA – ALTRI, Constância	**NS**	**60**[5]	**45**	**8**	**350**	**Isolate C4**	**12.2**	**0.04**	**0.39**	**75**	**(Henriques et al., 2018)**
	NS	60[5]	33	9	63	Adapted population	6.93	0.11	0.26	75	(Pereira et al., 2015)
		60[5]	39	9	92	Isolate C4	4.6	0.05	0.16	68	
Tembec, Témiscaming	NS[4]	100	34.1	10	50	GS301	1.8	0.036	-	< 20	(Bajwa et al., 2010)
Hindustan mill, Nagoan, Assam	20-22	100	37.7	9.3	96	(Adapted strain)	6.7	0.07	0.28	44	(Nigam, 2001)
						P. tannophilus					
Tembec, Témiscaming	NS[4]	70	43	5.3	48	GHW301	8.5	0.177	-	95	(Harner et al., 2015)
	NS[4]	80	43	6.1	48	GHW301	7.4	0.154	-	60	
						E. coli					
Tembec, Témiscaming[6]	**20-22**	**100**	**33.4**	**8.6**	**120**	**ATCC 11303**	**11.2**	**0.12**	**0.38**	**100**	**(Lawford and Rousseau, 1993)**

[5] Pre-treatment included adjusting HW-SSL to pH 7 with KOH, aeration with compressed air after which lignosulphonates and other colloids were removed by means of centrifugation and microfiltration

[6] Ethanol titre and volumetric productivity results were inconsistent for this study

Table 3: Most successful studies for ethanol production from non-detoxified HW SSL

Glucose (g/L)	Mannose (g/L)	Galactose (g/L)	Xylose (g/L)	Inoculum strength	Nutrients[1] (g/L)	pH	EtOH (g/L)	Ref
3.9	8.5	2.5	21.8	4 g/L	-	6.5	12	(Helle et al., 2004)
5	-	-	40	0.4 (OD)	YE – 2.5 DAP – 2 AS – 1 MSHH – 0.5	5.5	12.2	(Henriques et al., 2018)
2.4	8.6	-	22.4	0.5 g/L	Tryptone – 10 YE – 5	7	11.2	(Lawford and Rousseau, 1993)

The study by Helle et al. (2004) is one of the oldest studies to utilise xylose capable *S. cerevisiae* strains in the fermentation of HW-SSL. A high yeast concentration (4 g/L) was used to increase fermentation rate. Large inoculums can serve as inhibitor mitigating strategies as will be discussed in Section 2.4.2.3 but are often too expensive to implement on industrial scale. Furthermore, (Helle et al., 2004) utilised an initial pH 6.5 which decreased to 5.3 during the course of the fermentation. The initial high pH could further alleviate the effect of weak acids present in the SSL, which can lead to higher xylose consumption rates and ethanol productivity (Casey et al., 2010; Ko et al., 2020; Novy et al., 2013). The potential contamination risk by bacterial species at this pH was reduced by the large inoculum, thus increasing the ability of the ethanolic yeast to outcompete unwanted microbial species (Huang et al., 2011).

Henriques et al. (2018) indirectly utilised the effect of a high yeast density by implementing a two-stage aeration fermentation in which the acetic acid is consumed during aerobic fermentation in the first step without notable losses in sugar concentration. This led to a two-fold fermentation improvement, since acetic acid was then completely removed from the system, and the biomass concentration was increased to 6.47 g/L dry weight which is favourable when dealing with high inhibition mediums such as SSL. Another factor which could have improved the fermentation in this study is the pretreatment of the medium to remove

[1] Abbreviations are as follows: Yeast extract - YE; $(NH_4)_2HPO_4$ – DAP; $(NH_4)_2SO_4$ – AS; KH_2PO_4 – MKP; $MgSO_4{\cdot}7H_20$ - MSHH

the unfermentable solids. The SSL pH was increased to 7, and the precipitated colloids removed by means of centrifugation and ultrafiltration. The aim of this was to remove part of the lignosulphonates from the SSL, simplifying biomass determination, however it is likely that this pH-increase also caused the precipitation of phenolic compounds (Chandel et al., 2012; Jonsson et al., 2013). Phenolic compounds and lignosulphonates form part of the inhibitory compounds present in SSL, and their removal will likely lead to increased fermentation performance (Kim, 2018; Taherzadeh and Karimi, 2011). Although adjustment to pH 7 is not as effective as over-liming where the SSL is adjusted to pH 10 to precipitate inhibitors (Taherzadeh and Karimi, 2011), the neutralisation pretreatment to remove solids can be considered as a kind of detoxification. Pretreatment is however not economically desirable as it often requires additional process steps and chemicals, which leads to increased expenses.

Lawford and Rousseau, (1993) utilised a genetically modified *E. coli* strain for ethanol production from HW-SSL. pH 7 was maintained throughout the fermentation which is within the range pH 6.5-7.5 which is optimal for *E. coli* growth (Davey, 1994), which would also reduce the inhibitory effect of weak acids such as acetic acid (Lawford and Rousseau, 1993; Novy et al., 2013). Furthermore, the usage of laboratory nutrients such as yeast extract (5 g/L) and tryptone (10 g/L) likely promoted growth and ethanol production in inhibitor SSL, as nutrient rich mediums increase cell viability and reduce inhibitory effects (Helle et al., 2008; Jørgensen, 2009). These laboratory scale nutrients are however not economically feasible and should rather be replaced with an inexpensive nitrogen source such as corn steep liquor (Brandt, 2019).

In order to improve on the current results from literature in the fermentation of non-detoxified HW-SSL, one needs to achieve a minimum of 12 g/L ethanol while utilising low cell densities, inexpensive nutrients and refrain from utilising any pretreatment. The usage of modern novel xylose capable recombinant *S. cerevisiae* strains coupled with bioprocess techniques such as cell condition and fed batch strategy can lead to an improvement in ethanol production.

2.3 Microorganism

The selection of the correct microorganism strain for a specific feedstock is essential, since strains adapted for one type of feedstock is not always suitable for another (Kim, 2018). The optimisation of SSL fermentation should therefore always include a rigorous strain screening step (Modig et al., 2008).

2.3.1 Desired phenotypes

As previously mentioned, the main challenges associated with the fermentation of HW-SSL are the high inhibitor concentrations and inefficient xylose utilising ability of microorganisms. Therefore the key microorganism phenotypes required for HW-SSL fermentations are inhibitor tolerance and xylose utilising capabilities (Avanthi et al., 2017).

Robustness can either be improved by the adaptation of the microorganism to the toxic feedstock, thereby increasing tolerance to the inhibitors, or increasing the ability of cells to consume inhibitors as explained in Section. It is however essential that the microorganism is tolerant against a range of inhibitors present in SSL, since durability towards one inhibitor compound does not necessarily relate to other toxic (Brandt, 2019; Pereira et al., 2013; Wang, Sun, et al., 2018).

Efficient xylose consumption is also required in HW-SSL fermentations, since xylose constitutes 70-85% of the sugar content (Chipeta et al., 2005; Lawford and Rousseau, 1993; Rueda et al., 2015). This is a multifaceted challenge, as receptors, metabolic pathways and homeostasis regulators need to be optimised for xylose consumption by *S. cerevisiae*, which is not naturally capable of pentose fermentations (Bergdahl et al., 2012; Cunha, Romaní, et al., 2019). As mentioned in Section 2.1.4, the xylose capability phenotype is often more sensitive to inhibitors in recombinant strains than that of hexose capability. Cunha, Romaní, et al. (2019) states that one should select an already robust strain for the implementation of xylose capability to ensure that xylose can be fermented in lignocellulosic feedstocks. This implies that xylose affinity cannot be optimised without taking robustness also into consideration.

2.3.2 Microorganism species

The glucose fermenting baker's yeast, *S. cerevisiae*, is a popular microorganism in lignocellulosic fermentations due to its superior inhibitor- and ethanol tolerance compared to natural xylose fermenting yeast species (Bajwa et al., 2009; Hahn-Hägerdal et al., 2007; Pereira et al., 2013). *S. cerevisiae* strains genetically engineered towards xylose capability are also more efficient in the co-fermentation of glucose and xylose than naturally xylose fermenting yeast species (Helle et al., 2004; Nigam, 2001). Xylose capable *S. cerevisiae* strains are therefore the preferred microorganism for SSL fermentations, since the robustness of this species makes them more suitable for industrial applications (Hahn-Hägerdal and Palmqvist, 2000).

2.3.3 Recombinant *S. cerevisiae* strains

CelluX™1 (supplied by Leaf by Lesaffre, France) is a recombinant *S. cerevisiae* strain engineered with the XI-pathway for xylose consumption for usage in lignocellulosic fermentations; further improvements by means of adaptation, carried out within the company, produced the fourth generation of this strain namely CelluX™4. Brandt (2019) used CelluX™1 as parental strain and developed numerous derived strains, including TP1 and TFA7, that were hardened (improved inhibitor tolerance) by means of multiple rounds of rational engineering and screening techniques with simultaneous tolerance towards a range of inhibitors. Figure 3 displays the process by which these advanced recombinant strains has been engineered. TFA7 was developed from CelluX™1 by the overexpression of genes TAL1 (trans aldolase 1), FDH1 (formate dehydrogenase), ARI1 (aldehyde reductase) and ADH6 (alcohol dehydrogenase), which encodes for acetic acid tolerance and the increased ability to detoxify formic acid, furans, vanillin and cinnamaldehyde (Brandt, 2019). Apart from all the genes overexpressed in TFA7, TP1 was further improved by the introduction of genes PAD1 (phenylacrylic acid decarboxylase) and ICT1 (lysophosphatidic acid acyltransferase) which is associated with aromatic compound (ferulic and cinnamic acid) catabolism and tolerance towards organic solvents, respectively (Brandt, 2019).

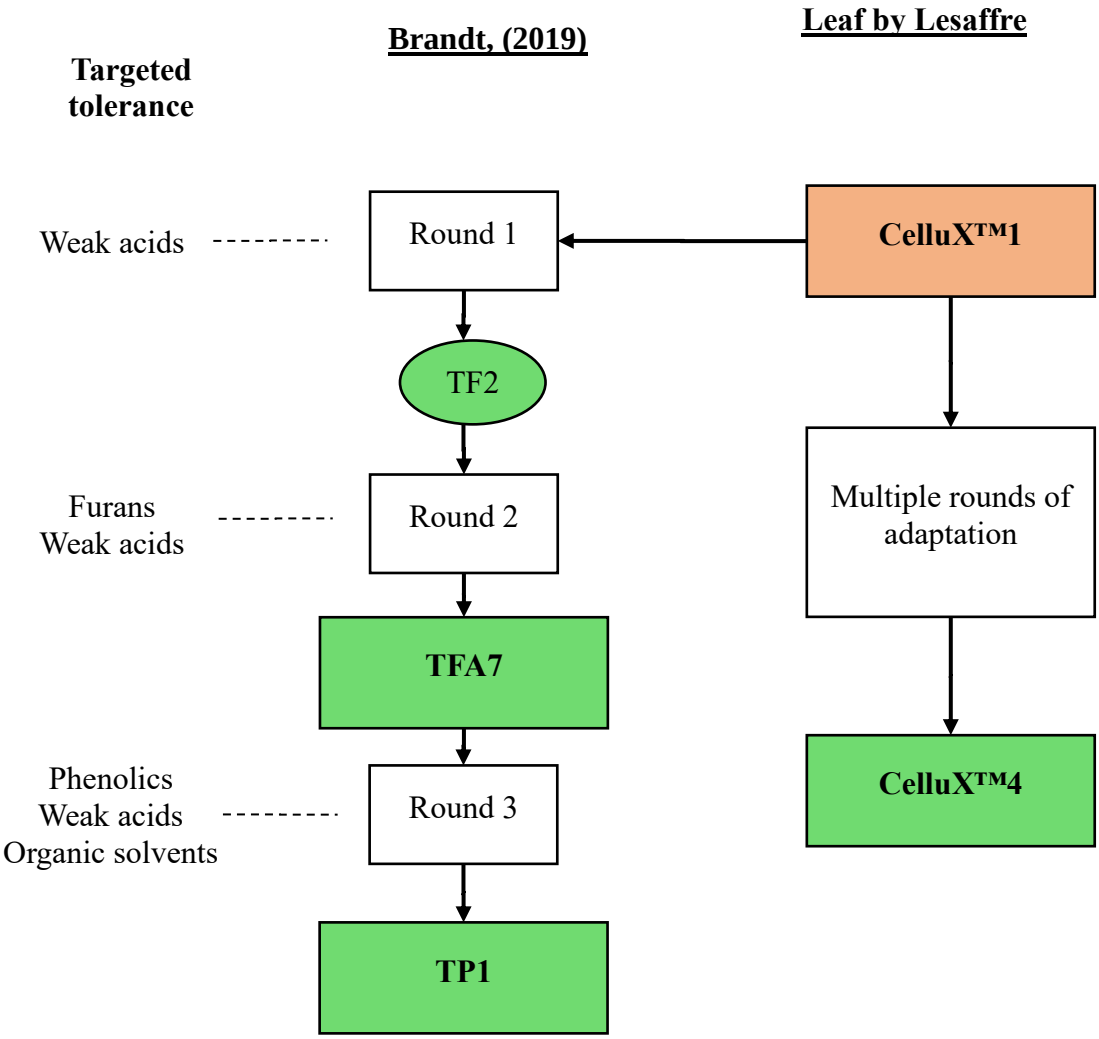

Figure 3: The development of robust, xylose capable recombinant *S. cerevisiae* strains for ethanol production in lignocellulosic materials

CelluX™4 which is the fourth generation version of the CelluX™1 strain, was develop by means of multiple rounds of adaptation. (Mokomele, 2019) found that, along with TP1, CelluX™4 could consume essentially all the xylose (30 g/L) and glucose (80 g/L) in hydrolysate from steam exploded sugar cane bagasse (StEx-SCB) in the presence of typical inhibitors such as HMF and furfural (0.65 g/L), acetic acid (8 g/L) and phenolic compounds (0.16 g/L). CelluX™4 and TP1 have also shown the ability to significantly decrease the concentration of the phenolic compounds by 30% and 25% respectively, and deplete the furfural and HMF in the StEx-SCB media (Mokomele, 2019). Table 4 compares the composition of SSL (Brandt, 2019) and StEx-SCB hydrolysate (Mokomele, 2019).

Table 4: Compositions of the substrates fermented using the advanced yeast strains

	Brandt (2019)	**Mokomele, (2019)**
Feedstock	**HW-SSL**	**StEx-SCB whole slurry**
Sugars (g/L)		
Glucose	14.7 ± 0.02	80.82 ± 2.21
Xylose	92.7 ± 1.03	33.71 ± 0.96
Organic acids (g/L)		
Acetic acid	15.1 ± 0.48	8.11 ± 0.43
Formic acid	0.56 ± 0.01	0.81 ± 0.07
Furans (mg/L)		
Furfural	2080 ± 0.0	651.33 ± 16.03
HMF	210 ± 10	66.00 ± 1.89
Phenolics (mg/L)		
Vanillic acid	116 ± 32.1	10.35 ± 0.073
Ferulic acid	275 ± 21.4	17.02 ± 1.22
Cinnamic acid	72.0 ± 18.0	-
Coumaric acid	-	60.23 ± 3.34
3,4-Dihydrobenzoic acid	46.10 ± 9.25	2.48 ± 0.61
3,5-Dihydrobenzoic acid	1050 ± 360	-
Syringic acid	308 ± 34.4	9.61 ± 3.51
Vanillin	76.1 ± 9.39	36.82 ± 1.21
Syringealdehyde	138 ± 14.1	14.38 ± 1.13
Coniferylaldehyde	15.9 ± 6.2	6.81 ± 0.81

Table 4 shows that HW-SSL contains higher phenolic compound and acetic acid concentrations than the hydrolysate used to compare TP1 and Cellux4™ performance as also confirmed by (Costa et al. 2017). There exists a markable difference between the two substrates in terms of phenolic compounds with HW-SSL containing 13-fold the amount present in SCB. SSL also contains 90% more acetic acid than SCB, with a concentration of 15 g/L. 5 g/L acetic acid is considered detrimental to xylose fermentation as displayed in Figure 2. HW-SSL also contains 80% less glucose than SCB, which renders SCB more fermentable as *S. cerevisiae* has a higher affinity to glucose (Moysés et al., 2016; Subtil and Boles, 2012). It is therefore to be expected that the novel strains might not perform as well in HW-SSL as in SCB. The employment of fermentation strategies discussed in Section 2.4 are critical in the attempt to maximise ethanol titre.

2.4 Improvement strategies

Fermentation of HW-SSL is not effective due to the lower xylose utilisation capability displayed by the strain in the presence of inhibitors. These challenges can be addressed by means of process improvement strategies, which can increase the ethanol yield, titre and volumetric productivity. However, many of these strategies are expensive to incorporate into the fermentations system, and it is necessary to weigh the ethanol increase benefit with the additional cost before implementation. Table 5 presents a summary of potential improvement strategies. Each strategy will be discussed in more detail in the upcoming sections.

Table 5: Summary of lignocellulosic improvement strategies and associated compromises on industrial fermentation goals

	Challenges		**Compromise on aims**	
Improvement strategies	**High inhibition**	**Low xylose consumption**	**High ethanol production**	**Low production cost**
Preculture detoxification	Decrease inhibition		Decrease sugar concentration	Additional chemicals and operations
Microbial *in situ* detoxification	Decrease inhibition			
Coculture	Decrease inhibition	Xylose utilising species		
Genetic engineering	Decrease inhibition	Xylose ability		
High inoculum density	Increase tolerance			Additional chemicals
Nutrient-rich media	Increase tolerance			Additional chemicals
Medium dilution	Decrease inhibition		Decrease sugar concentration	
Microbe conditioning	Increase tolerance	Activate xylose metabolism		
Fed batch strategy	Decrease inhibition	Increased xylose metabolism		

2.4.1 Pre-fermentation detoxification

SSL can be detoxified by means of physical, chemical and biological methods before fermentation. Amongst others these methods include alkali treatment, adsorption, evaporation, stripping, liquid-liquid-extraction and the addition of chemical reducing agents and enzymes (Alriksson et al., 2011; Jonsson et al., 2013; Llano et al., 2015, 2017).

However, there are many shortcomings with regards to detoxification on an industrial level. Detoxification will often require a separate step in the process, as is the case with adsorption, stripping, liquid-liquid-extraction, alkali treatment and biological treatment by another microorganism, which has a negative effect on economic feasibility (Holderby and Moggio, 1960; Kim, 2018; Rivard et al., 1996). Even *in situ* treatment methods that do not required an additional step in the process, such as chemical reducing agents (Alriksson et al., 2011) and enzyme addition (Jönsson et al., 1998), will require the use of additional materials. Enzyme usage may be more cost effective if preserved by immobilisation techniques (Chandel et al., 2012), but this will add complexity and cost to the process.

Fermentable sugars are also often lost in the detoxification steps such as alkali (Nilvebrant et al., 2003) and biological (Chandel et al., 2012; Pereira et al., 2012) treatments. In neutralisation by alkali treatment some sugar precipitates out with the inhibitors, whereas the detoxifying microorganisms consume a part of the sugar in biological treatment (Kim, 2018). Overliming (neutralisation with $Ca(OH)_2$) has the additional challenge of gypsum precipitation that needs to be handled downstream (Martinez et al., 2001). Adsorption methods will either result in waste formation, or increased production cost if resins are regenerated (Chandel et al., 2012).

Due to the expenses and added complexity, preculture detoxification should only be considered if deemed necessary. The efficiency and limitations of other mitigating strategies yet to be discussed, such as *in situ* detoxification and advanced feeding strategies should be investigated first, as these or a combination can be sufficient to alleviate inhibition (Johansson et al., 2011; Pereira et al., 2013; Zhang et al., 2014).

2.4.2 Microbial *in situ* detoxification

Some wild-type microbial strains possess detoxification properties that enable them to convert inhibitory compounds to less harmful substances (Chandel et al., 2012; Taherzadeh and Karimi, 2011). These capabilities can be optimised by means of genetic engineering and process improvement strategies. Table 6 summarises the use of different biological detoxification methods and the simplification of the process by means of combining steps.

Table 6: The combination of process steps in lignocellulosic liquid fermentations by means of recombinant microbial improvements [1]

	Biological detoxification	**Glucose fermentation**	**Xylose fermentation**
Preculture detoxification			
Coculture fermentation	Preculture detoxification by a different species	Glucose capable strain	Xylose capable strain
Monoculture co-fermentation	Preculture detoxification by a different species	Co-fermenting strain **(Genetic engineering)**	
In situ detoxification			
Enzymatic *in situ* detoxification	Addition of external enzymes into culture	Co-fermenting strain **(Genetic engineering)**	
Coculture fermentation	Detoxifying glucose capable strain		Xylose capable strain
Monoculture co-fermentation	Detoxifying co-fermenting strain **(Genetic engineering)**		
High cell density	**Improved robustness and xylose fermentation**		

Although preculture microbial detoxification is considered more economical and less energy intensive than physical and chemical pretreatment steps, the strategy not only leads to a separate process step, but also a loss of sugars (Kim, 2018; Pereira et al., 2012). Alternatively, it is possible to add enzymes such as laccase or peroxidases to degrade inhibitors into the fermentation to facilitate *in situ* detoxification (Chandel et al., 2012; Jönsson et al., 1998). This external enzyme addition will however add to the cost of the fermentation.

The ideal case for biological detoxification is the development and utilisation of microorganisms capable of removing inhibitors, while simultaneously producing ethanol (Wang, Li, et al., 2018). This will lower production expenses by eliminating the cost of pretreatment and/or additional enzymes and reducing the number of process units.

[1] Solid lines represent separate steps in the process whereas dotted lines represent different components in one process step

2.4.2.1 Coculture

It has been shown before that ethanol production can be improved in lignocellulosic fermentations by using multiple microorganism strains or species (Soares et al., 2017; Zhang et al., 2014). The typical mechanism of symbiosis entails the use of strains that target different sugars (typically xylose and glucose) such that no competition occurs within the culture. Recently it has been shown that this effect can further be exploited if a robust *S. cerevisiae* strain, capable of glucose consumption and *in situ* detoxification, is used alongside a less robust xylose capable microorganism from the same or different species (Zhang et al., 2014; Zhu et al., 2016). A heterogenous population can offer the opportunity for symbiotic roles to become more defined (Pereira et al., 2015). Coculture strategy also has the advantage of making it possible to optimise the functions of each microorganism independently, without compromising the other function (Liu et al., 2020).

As will be discussed in the next section however, the continuous improvement of recombinant strains can be efficient in combining the desired phenotypes of a coculture into a single microorganism.

2.4.2.2 Genetic engineering

S. cerevisiae naturally produce enzymes capable of detoxifying a range of inhibitors within the furan and phenolic groups (Wang, Li, et al., 2018). By overexpressing or inserting genes to increase production of these enzymes in *S. cerevisiae* strains, the detoxification ability of the cells can be increased. When this is coupled with recombinant xylose capable strains, the process becomes simpler as the need for coculture is eliminated. In the past recombinant xylose capable strains have been modified to rid fermentation media of furfural, HMF, acetic acid and certain phenolics (Brandt, 2019; Cunha, Romaní, et al., 2019; Zhang et al., 2016). It is however important that the improvement of tolerance due to detoxification ability should not lead to a metabolic burden on the microorganisms (Wang, Sun, et al., 2018).

2.4.2.3 Inoculum size

In fermentations where cell growth is impeded and carbon is not easily directed away from ethanol production, large inoculums have been shown to disproportionately improve fermentation of lignocellulosic materials as it not only increases productivity as expected, but also results in higher yield (Helle et al., 2008; Ko et al., 2020). The tolerance and *in situ* detoxification ability of the yeast strains are also improved by larger inoculums, implying that high cell pitch is an inhibitor reduction strategy (Cantarella et al., 2004; Chung and Lee, 1985;

Pienkos and Zhang, 2009). Multiple inoculations throughout fermentation can also serve a detoxification purpose as cell regrowth leads to inhibitor consumption (Zhang et al., 2014).

However, the nutrients to grow the inoculum can become a large production expense and maintenance of a vast amount of cells are not always practical, and is therefore not seen as an industrially viable solution (Helle et al., 2008; Wingren et al., 2003). The benefit of a higher cell-to-substrate ratio can be obtained by using a fed batch strategy, as will be discussed in more detail in Section 2.4.5.2.

2.4.3 Nutrient rich media

The addition of nutrients during SSL fermentation significantly increases ethanol production as inhibitor tolerance can be improved and cell viability and growth sustained (Helle et al., 2008; Johansson et al., 2014; Taherzadeh and Karimi, 2011). Lab scale nutrient sources such as peptone and yeast extract are most efficient at improving fermentation of lignocellulosic materials (Helle et al., 2008; Jørgensen, 2009). These substances are however not industrially feasible, as they are too expensive. More economic alternatives such as corn steep liquor (CSL), though not as effective, has been shown to also improve fermentation performance and should therefore rather be utilised (Brandt, 2019; Helle et al., 2008; Jørgensen, 2009).

2.4.4 Microbe conditioning

The fermentation performance of microorganisms is dependent on the conditions that the organism is subjected to during inoculum preparation (Alkasrawi et al., 2006; Chandel et al., 2012). One way to improve fermentation performance is by including lignocellulosic media in the cultivation medium used for inoculum preparation. The microorganism is gradually conditioned to the inhibition stress associated with SSL media, thus allowing the cells to better maintain viability during the fermentation which can be related to increased ethanol productivity, titre and yield (Alkasrawi et al., 2006; Johansson et al., 2011). In the case of a high xylose material such as HW-SSL, the inoculum should also be prepared in media containing a high xylose concentration which enables the activation of xylose metabolising pathways (Brandt, 2019; Mokomele, 2019). Not only does this enable the activation of required metabolic pathways to decrease lag phase and increase productivity (Shuler et al., 2017), but the improved tolerance of the microorganism results in higher ethanol yields (Johansson et al., 2011).

2.4.5 Fed batch strategy

Fed batch strategy has long been applied to the fermentation of lignocellulosic feedstocks as it enables microorganisms to mitigate the effects of high inhibitor concentrations (Taherzadeh and Karimi, 2011). Amongst others, feeding can improve cell conditioning, *in situ* detoxification and possibly enhance co-fermentation abilities. To the author's knowledge, fed batch strategy has not yet been applied to HW-SSL fermentations.

2.4.5.1 Microbe conditioning

As discussed earlier in Section 2.4.4 on microorganism conditioning, it is possible to increase the fermentation yield, titre and volumetric productivity by growing the yeast inoculum in a partially lignocellulosic medium, containing both the sugars and inhibitors present in SSL media (Alkasrawi et al., 2006). If fed batch fermentations are started at a sufficiently low inhibitor concentration, feeding can also serve as a method of acclimating microorganisms to the toxic media. Successful fermentations have been carried out on toxic media by means of substrate feeding, that have been unfermentable in batch cultures (Taherzadeh et al., 1999). The abrupt exposure to harsh conditions in a batch culture can cause a shock in microorganisms, as high inhibitor concentrations tend to affect cell viability negatively (Johansson et al., 2011). Gradual introduction of inhibitors by means of fed batch strategy, might ensure that cell viability stays high for longer, enabling conditioning of cells and also improving the overall fermentation performance.

2.4.5.2 Increased *in situ* detoxification

Fed batch fermentation can act as a strategy to increase the ability of strains to remove the inhibitors (Parawira and Tekere, 2011; Pereira et al., 2013; Soares et al., 2017). The detoxification ability of yeast strains is usually only effective below a certain inhibition threshold, implying inhibitors cannot be sufficiently removed in a too toxic media (Zhang et al., 2014). By feeding the substrate slowly, the inhibitor concentrations remain at levels where they can be consumed or converted to less toxic compounds more effectively, and ideally keep it below the lethal threshold concentration (Zhang et al., 2014; Zhu et al., 2016). Feeding also ensures that the initial biomass concentration is higher than it would be in an equal batch culture, since the starting volume is smaller in fed batch fermentations. Higher cell density can lead to increased tolerance and *in situ* detoxification abilities in strains as discussed in Section 2.4.2.3. A combination of lower inhibitor concentrations and higher cell density can therefore increase the ability of cultures to consume inhibitors.

In media where the toxicity levels are too severe, inhibitors cannot always be removed and fermentations will still reach a premature stagnation point (Taherzadeh and Karimi, 2011). In such cases feeding can still allow a head start for cells to increase performance in terms ethanol production at low inhibitor concentrations. By delaying the time at which severe toxic levels are reached, ethanol concentration can be improved compared to that of batch cultures, which were already started at the lethal inhibitor concentrations.

2.4.5.3 Enhanced co-fermentation ability of glucose and xylose

Fed batch strategies can also be implemented to avoid catabolite repression and adjust metabolic pathways (Shuler et al., 2017). In the case of *S. cerevisiae*, even strains genetically engineered and adapted for xylose consumption, have a much higher affinity for glucose (Avanthi et al., 2017). In these fermentations, xylose will often only be consumed once all the glucose is depleted, since glucose and xylose are competing for the same transporters into the cells and *S. cerevisiae* prefers glucose (Helle et al., 2008; Moysés et al., 2016; Subtil and Boles, 2012). Since the xylose concentration in HW-SSL is significantly higher than that of glucose (Helle et al., 2004), it is imperative to maximise xylose consumption. Co-fermentation of glucose and xylose can only be achieved at glucose limiting conditions, with xylose consumption being suppressed at glucose concentrations of 20 g/L and above (Avanthi et al., 2017). It has however been found that glucose concentrations of 0-2 g/L can stimulate xylose consumption by activating sugar receptors to enhance xylose transport into the cells (Meinander and Hahn-Hägerdal, 1997; Souto-Maior et al., 2009). Hexose repression can therefore be prohibited, while xylose uptake can even be increased if substrate is fed at a rate to keep glucose concentrations below 2 g/L.

2.4.5.4 Optimisation of feed rate

Optimisation of the feeding profile is required since there exists a trade-off between productivity and yield at different dilution rates (Han et al., 2017). A slow feeding rate leads to a low ethanol volumetric productivity since the fermentation time is longer but can simultaneously increase the ethanol yield since inhibitors are added slower. Too fast addition of substrate can prevent efficient cell conditioning or *in situ* detoxification, which will in turn lead to a drop in ethanol yield and titre (Taherzadeh and Karimi, 2011; Zhang et al., 2014). An exponential feed rate is preferable over a constant volumetric feed rate as the former can lead to higher ethanol yield and productivity (Cofré et al., 2016; Han et al., 2017; Karapatsia et al., 2016).

The optimisation of HW-SSL feeding for ethanol production has not been investigated, according to the author's knowledge. It remains to test the effect of feed rates and profile and determine if the expected improvements on fermentation performance can be observed.

2.5 Gap in literature

Advancements on previous literature studies

The highest ethanol titres obtained from non-detoxified HW-SSL in past studies in literature, is in the range of 11.1-12.2 g/L. These studies utilised a combination of strategies such as large inoculums (5-6 g/L), high pH (6.5-7), expensive nutrients and pretreatment steps namely treatment with KOH (pH 7), stripping and microfiltration to decrease inhibitor effects and improve fermentation performance. Although these methods are effective for laboratory experiments, these strategies are not industrially relevant due to increased processing expenses. The goal for industrial implementation is a process that obtains ethanol titres higher than 12 g/L from non-detoxified HW-SSL (as is), while utilising a yeast inoculum concentration below 1 g/L dry weight, inexpensive nutrient sources such as CSL and operating conditions of pH 5.

Usage and comparison of new robust and xylose capable recombinant *S. cerevisiae* strains

Although the usage of xylose capable recombinant yeast strains is not novel in lignocellulosic derived feedstock or even SSL fermentations, genetic engineering continuously offers improved yeast strains. The industrial strains CelluX™4 has been successfully implemented in the fermentation of other lignocellulosic media within the research group, but usage of this novel strain in HW-SSL fermentations is not available in literature. The robustness and xylose capability of this strain makes it a prime candidate for a challenging substrate such as HW-SSL. The comparison of CelluX™4 to the hardened strain TFA7 further adds novelty to the present study.

Implementation and variance of fed batch strategy in HW-SSL fermentation

To the best knowledge of the author, fed batch strategy has not yet been implemented in ethanol production processes where HW-SSL is used as fermentation medium. The studies where fed batch culture has been used in the fermentation of other lignocellulosic derived substrates, illustrates the potential of this strategy to mitigate inhibition and improve ethanol yield and titre.

2.6 Research questions

I. Which SSL stream is most suitable for usage in ethanol production?

Various SSL concentrates emanate from the multistage evaporator. These streams differ in solid concentration, sugar and inhibitor content. In order to achieve economically feasible concentrations of ethanol, high TDS SSL from the later stages in the evaporator will most likely be used as opposed to more dilute SSL before concentration, as these contain a sufficiently high sugar concentration.

Literature sources are divided on whether inhibitor concentrations are increased or decreased in the evaporator system. This study will determine whether there is a sugar-inhibitor trade-off in the concentrations of streams. If a low TDS SSL stream is not fermentable, as it contains similar inhibitors to that of a high TDS stream, it might be more beneficial to dilute the high TDS stream with water, thus lowering the inhibitor concentration to sub-lethal levels.

II. Which yeast strain is most suitable for ethanol production in a fed batch SSL fermentation?

The complexity of SSL as fermentation medium demands the use of an advanced microorganism to maximise ethanol production. In order to find the most suitable strain for HW-SSL fermentation, the novel strains must be evaluated in terms of the desired phenotypes. The most suitable strain will be required to display both inhibitor tolerance and xylose capabilities, as these are the prominent challenges in HW-SSL fermentations.

III. How does the feeding of a concentrated SSL stream in a fed-batch culture influence the ethanol yield and productivity compared to that of a batch fermentation?

It is expected that feeding of SSL should mitigate the effect of inhibition on yeast cells, based on the improvements seen in other feedstocks discussed in literature. By feeding the toxic media slowly, ethanol production can be maximised at the initial low inhibitor concentrations, whereas batch cultures are already initiated at high toxic levels. This gradual feeding also allows time to condition cells to higher inhibitor levels while inhibitors are removed by means of *in situ* detoxification by the yeast. The fed batch process can be further improved by optimising the dilution rate and increasing the inoculum size. This study will identify which process performance improvements, such as to increase ethanol concentration from non-detoxified HW-SSL, can be achieved with this method.

IV. How does the maximum ethanol titre obtained under harsh industrial conditions, using novel xylose capable recombinant *S. cerevisiae* strains, coupled with fed batch strategy, compare to that of other literature studies where non-detoxified HW-SSL was fermented?

Due to high inhibitor and low hexose concentrations in HW-SSL and the current challenges with the fermentation of xylose in such toxic media, only three studies have been able to obtain more than 10 g/L (11.2-12.2 g/L) of ethanol. The usage of novel strains such as CelluX™4 and TFA7 in a fed batch fermentation is expected to achieve higher concentrations than that of literature sources. However, this study prioritised the implementation of strict industrially relevant conditions such as low cell density, pH 5, inexpensive nutrient sources and no pretreatment/detoxification of the SSL prior to fermentation, which will lead to harsher conditions than utilised by the most successful studies in literature.

CHAPTER 3: RESEARCH DESIGN AND METHODOLOGY

3.1 Experimental approach

The experimental approach followed in this study started out with the selection of an appropriate SSL stream following chemical characterisation. Thereafter different yeast strains were compared in SSL fermentations, followed by the investigation of performance improvements through the utilisation of fed batch strategy.

3.1.1 SSL stream selection - Characterisation

Chemical characterisation by means of HPLC was done on all the SSL streams to determine the sugar and inhibitor content and subsequently select suitable streams for future runs. The details of the characterisation are discussed in Section 3.3.

3.1.2 Shake flask batch fermentations

Preliminary experiments were conducted in shake flask batch cultures to assess the performance of different yeast strains in varied fermentation media. Strains were evaluated and selected according to obtained ethanol titre and productivity, which were in turn affected by the xylose utilisation and inhibitor tolerance capabilities of the microorganisms. The varying of SSL concentration also served as a screening procedure to determine a suitable starting SSL concentration for fed batch fermentations.

Figure 4: Shake flask fermentations under microaerophilic conditions, ensured by rubber stoppers and s-shaped airlock, carried out in an incubator

3.1.3 Fed batch concept

3.1.3.1 Pulse addition fed batch cultures

Fed batch strategy was conceptually tested by means of pulse additions of high TDS SSL into shake flask cultures. The culture was started at a low SSL concentration and increased with the periodic pulsing of undiluted concentrated SSL during the fermentation. The best performing yeast strain was used to ultimately reproduce the fed batch fermentation in 5L bioreactors with gradual feeding of SSL. The xylose consumption rate observed during the batch phase of the best performing fermentation was used to estimate the maximum permittable SSL feeding rate for the bioreactor runs.

3.1.3.2 Gradual addition fed batch cultures

Fermentations were carried out in 5L bioreactors as described in Section 3.7 to assess the influence of gradual SSL addition on ethanol production.

Figure 5: Fed batch fermentations carried out in a bioreactor.

3.2 HPLC analysis

Ethanol, glycerol, glucose, xylose, acetic acid, formic acid, lactic acid, furfural and HMF were analysed on an HPLC instrument (Thermo Separations Product) fitted with a 250 x 7.8mm Biorad HPX-87H column, with guard cartridge. The column was operated at 65°C with 5 mM H_2SO_4 at a flowrate of 0.6 ml/min as mobile phase. Peak detection was performed with a Shodex 101-RI detector for all the compounds except the furans where a UV detector (280 nm) was used.

The phenolic compounds were analysed on a Dionex 3000 System with UV detector (285 nm) fitted with a 250 x 4.6mm XSelect C18 column. The column was operated at 37°C with two gradients as eluents, namely water-TFA and acetonitrile-TFA, at a flowrate of 0.7 ml/min.

3.3 Feedstock characterisation

The liquid samples were characterised by high performance liquid chromatography (HPLC) analysis to determine the sugar and inhibitor content. The total dissolved solids (TDS) content was determined through dry weight as discussed in Section 3.3.3.

3.3.1 Source

Eight different HW-SSL streams were sampled from the Sappi Saiccor mill (Umkomaas, KwaZulu-Natal). The samples consisted of one dilute stream from the CaO cooking line and seven streams from the MgO cooking line. The MgO SSL streams were collected from sampling ports at different stages in the multi-effect evaporator system.

3.3.2 Chemical characterisation

All the chemical compounds described in Section 3.2 were quantified for all eight SSL streams.

3.3.3 Total dissolved solids

The total dissolved solids (TDS) content was analysed by drying samples from each SSL stream. A 2 g liquid sample ($m_{wet\ SSL}$) was inserted into a small foil container of known mass. Thereafter the foil containers were placed in a drying oven at 50°C until no further mass change was observed, which occurred after approximately 72 h. Samples from all SSL streams were analysed in triplicate. Equation **(1)** was used to calculate the dissolved solid content.

$$\%\,TDS = 100\% \cdot \frac{m_{foil\ post-drying}(g) - m_{empty\ foil}(g)}{m_{wet\ SSL}\ (g)} \qquad (1)$$

3.4 Microbial strains

Three xylose-fermenting (XI-pathway) recombinant *S. cerevisiae* strains, namely CelluX^{TM}4, TP1 and TFA7, were used in the study. TP1 and TFA7 are hardened strains developed by Brandt (2019), from the industrial strain CelluX^{TM}1 (Leaf by Lesaffre), through a sequence of genetic engineering steps and strain screening designed to improve tolerance towards a variety of microbial inhibitors. TFA7 received overexpression of genes TAL1, FDH1, ARI1 and ADH6 which encodes acetic acid tolerance, and enhanced catabolism ability of formic acid, furans, vanillin and cinnamaldehyde. Besides the genes overexpressed in TFA7, TP1 further received genes PAD1 and ICT1 which encodes for aromatic compound catabolism (ferulic and cinnamic acid) and tolerance towards organic solvent, respectively. CelluX^{TM}4 is the fourth-generation strain of CelluX^{TM}1 and will be used as industrial reference strain.

3.5 Inoculum preparation

The yeast strains were stored as glycerol stocks at -80 ºC. A week before the start of the fermentation the cells were streaked out on 2% YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) agar plates and incubated at 30 ºC until colonies formed.

The pre-inoculum was prepared by transferring cells from the agar plate into a cotton stoppered 250ml smooth Erlenmeyer flask containing 50 ml 2% YPD and incubated at 30 ºC and 150 rpm. After 24h, 10 ml of this pre-inoculum was used to inoculate 90 ml 25%-YPX-SSL for a total volume of 100ml to condition the yeast cells to the inhibitors present in SSL. 25%-YPX-SSL consists of 75 ml 2% YPX (xylose supplement instead of glucose) mixed with 25 ml high TDS SSL from the second last evaporator. SSL was adjusted to pH 5 prior to mixing by dissolving KOH pellets directly into the liquid. Conditioning was also done in a 250 ml cotton stoppered, baffled Erlenmeyer flask at 30 ºC and 150 rpm. YPD and YPX-SSL media were autoclaved at 121 ºC for 15 minutes prior to introduction of yeast cells.

3.6 Shake flask fermentations

3.6.1 Batch fermentations

Fermentations were conducted using synthetic medium or different concentrations of Mg-1A SSL from the second last evaporator stage, where the SSL was diluted with R/O water. Synthetic medium, designed to replicate the sugar composition of 40% SSL Mg-1A, was prepared by adding xylose and glucose into R/O water to a concentration of 30 g/L and 5.4 g/L respectively.

SSL and synthetic medium were adjusted to pH 5 by dissolving KOH pellets directly into the liquid. SSL was pH adjusted prior to dilution with R/O water to desired SSL concentration.

Corn steep liquid (CSL) was added as nitrogen source at a concentration of 5 g/L unless mentioned otherwise. Citric acid and sodium nitrate were added as buffer medium in concentrations of 16 mM and 34 mM respectively to maintain pH 5 during the fermentation.

Batch shake flask fermentations were carried out in 250ml rubber stoppered baffled Erlenmeyer flasks with a working volume of 100ml and incubated at 30 °C and 150 rpm. The media was sterilised at 121 °C for 15 minutes prior to inoculation. A 10% inoculum of the medium described in Section 3.5 was used, with an initial cell density of 0.65-0.84 g/L dry weight.

3.6.2 Pulse fed batch fermentations

Fed batch pulse fermentations were also carried out in 250ml rubber stoppered baffled Erlenmeyer flasks, incubated at 30 °C and 150 rpm. The inoculum was prepared using the same YPD, YPX-SSL method described for the batch fermentations. An inoculum volume of 10 ml was added into the fermentation medium to obtain a 70 ml initial working volume with 20% SSL concentration and cell density of 0.93-1.2 g/L dry weight. CSL was added at a concentration of 10 g/L based on the initial working volume of 70 ml.

The flasks were incubated for 48 h, as a batch phase, before the culture working volume was increased through the addition of undiluted SSL by means of pulses. Two-pulse and four-pulse feeding schemes were conducted in which the former consisted of two 24 ml pulses of high TDS SSL every 48 h and the latter of four 12 ml pulses every 24 h. The feeding schemes were designed to increase the high TDS SSL concentration of the broth from 20% to 60% throughout the fermentation.

3.7 Large scale bioreactor experiments

The fed batch process was started off with an initial SSL concentration of 20% (Mg-1A SSL diluted with R/O water) and a 2L working volume including the inoculum. Undiluted high TDS SSL was added continuously by means of a peristaltic pump until the final working volume of 4.6 L was reached to obtain a final SSL concentration of 65%. The inoculum broth constituted 15% of the starting volume of the fermentation unless otherwise stated, with an initial biomass concentration of 0.9 g/L dry cell weight.

Batch fermentations were started with a working volume and of 4.6 L where the Mg-1A SSL was diluted with R/O water to an SSL concentration of 65%. The same inoculum volume was added in the batch culture as in fed batch fermentations, implying an initial biomass concentration of 0.4 g/L dry cell weight for batch fermentations.

High TDS SSL from the second last evaporator stage was adjusted to pH 5 by directly dissolving KOH pellets into the liquid at a concentration of 20 g/L prior to fermentation. Batch and fed batch fermentations were carried out at 30°C and 200 rpm in 5L bioreactors (Sartorius). pH 5 was maintained by a closed control loop with continuous on-line pH measurement and addition of 3M KOH. 5 g/L CSL was added in batch and fed batch cultures based on the final working volume of 4.6 L. CSL was sterilised at 121 °C for 15 min and added to the reactor upon inoculation. The reactor and SSL were not autoclaved in order to maintain industrially relevant unsterile conditions.

3.8 Statistical analysis

Statistical analysis was conducted using the one-way analysis of variance (ANOVA) tool from MS Excel along with a Bonferroni procedure for *P*-value adjustment. A *P*-value of 0.05 was taken to indicate statistically significant difference between variables. All fermentations were performed in triplicate and representative results are given.

3.9 Fermentation performance analysis

All the chemicals mentioned in Section 3.3.2, except phenolic compounds, were also tracked throughout the fermentation. Sugars and ethanol concentrations were used to calculate fermentation parameters to compare different variables. Table 7 displays the main parameters calculated in this study.

Table 7: Fermentation parameters calculations

Parameter	Calculation	Unit
Ethanol yield ($Y_{P/S}$)[1]	$\frac{[EtOH\ produced]\ (\frac{g}{L})}{[Sugar\ consumed]\ (\frac{g}{L})}$	g/g
Ethanol productivity[2]	$\frac{[EtOH\ produced]\ (\frac{g}{L})}{Time}$	g/L·h
Xylose consumption rate[10]	$\frac{[Xylose\ consumed]\ (\frac{g}{L})}{Time}$	g/L·h
% Xylose consumption	$100\% \cdot \frac{[Xylose\ consumed]\ (\frac{g}{L})}{Xylose_i\ (\frac{g}{L})}$	%

Productivity was calculated by dividing the maximum ethanol concentration obtained by the fermentation time up to that point. Xylose consumption rate and inhibitor detoxification rate was calculated by dividing the consumed concentration by the fermentation time.

[1] Calculated graphically by plotting ethanol produced against sugar consumed and determining the slope of the straight-line section. Sugar and ethanol amounts were quantified as concentration (g/L) in batch fermentations and mass (g) in fed batch fermentations respectively.
[2] The fermentation time is complete when the increase in EtOH concentration is less than 5% of the previous sampling point

CHAPTER 4: RESULTS AND DISCUSSION

4.1 SSL characterisation – the feedstocks

The pulp and paper industry use multistage evaporation to remove water from SSL streams in preparation for incineration. The sugar and inhibitor contents of the intermediate streams in the multistage evaporator were determined by HPLC characterisation, as it plays a vital role in fermentation performance. Stream selection is primarily based on the ability of streams to meet a required ethanol titre of 40 g/L, which is dependent on the sugar concentration (Ogden et al., 1990). Since literature is divided on whether evaporators increase or decrease inhibitor concentrations, it is necessary to measure the inhibitor concentrations in all streams in case a less concentrated stream is more suitable for a batch phase due to lower toxicity. Table 8 displays the chemical composition of the SSL streams where Ca-SSL is from the CaO cooking line, and Mg-5 to Mg-out from the MgO-SSL evaporation line in increasing concentration. Mg-out is concentrated to the point where it is suitable for incineration and is therefore a very viscous liquid.

The SSL shows a four-fold increase in TDS from Mg-5 to Mg-out, indicating a removal of more than 90% of the moisture from the entry stream (Table 8). The pH values measured for all the streams in this study are within a low range of pH 2.3-3.0, indicating the need for pH adjustment prior to fermentation (Table 8). The pH values found in literature found in literature are within the range of 1.8-3 for SSL streams before and after evaporation (Chipeta et al., 2005; Marques et al., 2009; Rueda et al., 2015), which corresponds to that obtained in this study.

Table 8: Composition of SSL streams from the CaO cooking line and MgO evaporation line with Mg-5 to Mg-out in increasing thickness.

	Ca-SSL[1]	Mg-5 SSL	Mg-4 SSL	Mg-3 SSL	Mg-2 SSL	Mg-1C SSL	Mg-1A SSL	Mg-out SSL
TDS[2] (mass %)	16.8 ± 0.01	17.6 ± 0.14	26.3 ± 0.17	30.6 ± 0.15	31.5 ± 0.23	40.6 ± 0.07	54.2 ± 0.24	71.2 ± 1.79
pH	2.25	2.36	2.86	2.92	2.69	2.93	2.98	2.97
Sugars (g/L)								
Glucose	3.48 ± 0.04	3.88 ± 0.04	5.92 ± 0.22	6.88 ± 0.14	7.26 ± 0.10	9.72 ± 0.13	14.1 ± 0.05	21.6 ± 1.45
Xylose	30.6 ± 0.48	27.9 ± 0.58	39.8 ± 1.34	47.9 ± 0.27	51.7 ± 0.70	69.4 ± 1.93	93.1 ± 3.85	139 ± 8.25
Weak acids (g/L)								
Acetic acid	14.6 ± 0.14	14.1 ± 0.50	15.0 ± 0.41	16.0 ± 0.14	16.7 ± 0.06	16.4 ± 0.05	17.2 ± 0.31	15.3 ± 0.38
Formic acid	2.20 ± 0.04	< 0.67	< 0.67	< 0.67	< 0.67	1.18 ± 0.02	< 0.67	0.71 ± 0.06
Furans (g/L)								
Furfural	0.167 ± 0.005	0.615 ± 0.001	0.212 ± 0.004	0.167 ± 0.007	0.182 ± 0.007	0.124 ± 0.002	0.164 ± 0.004	0.409 ± 0.007
HMF[3]	< 0.034	< 0.067	< 0.067	< 0.067	< 0.067	< 0.067	< 0.067	< 0.067
Phenolics (mg/L)								
Ferulic acid	3.3 ± 0.2	4.0 ± 0.3	4.8 ± 0.8	5.6 ± 1.4	5.0 ± 1.9	12.7 ± 1.1	10.2 ± 1.8	8.9 ± 0.3
Syringaldehyde	12.4 ± 1.6	18.0 ± 0.1	16.3 ± 3.0	20.9 ± 0.6	18.8 ± 4.0	27.2 ± 4.6	46.3 ± 0.8	23.2 ± 2.4
Vanillin	1.8 ± 0.1	3.3 ± 1.1	5.1 ± 0.9	4.4 ± 0.6	7.0 ± 0.4	8.8 ± 0.9	14.7 ± 1.4	8.3 ± 1.3
Syringic acid	26.1 ± 1.3	28.1 ± 0.7	40.2 ± 1.7	50.4 ± 0.8	56.2 ± 3.6	74.1 ± 1.6	102.9 ± 7.8	79.6 ± 9.6
Vanillic acid	11.6 ± 0.3	13.5 ± 0.0	19.6 ± 0.4	23.3 ± 0.7	27.0 ± 0.0	36.7 ± 1.3	66.1 ± 12.0	50.8 ± 14.1
3,4 DHBA[4]	1.8 ± 0.3	12.6 ± 0.9	21.0 ± 2.0	26.9 ± 0.2	33.4 ± 10.0	49.5 ± 15.2	59.7 ± 4.9	30.9 ± 2.4

[1] Watery SSL from the CaO cooking line
[2] Total dissolved solids
[3] 5-Hydroxymethylfurfural
[4] Dihidrobenzoic acid

4.1.1 Sugar content

Evaluating the sugar content over various evaporator stages provide insight into sugar degradation and potential ethanol concentrations. Figure 6 is a visual representation of the data in Table 8 displays the relationship between the sugar concentrations and the TDS content of SSL streams across the different evaporator stages.

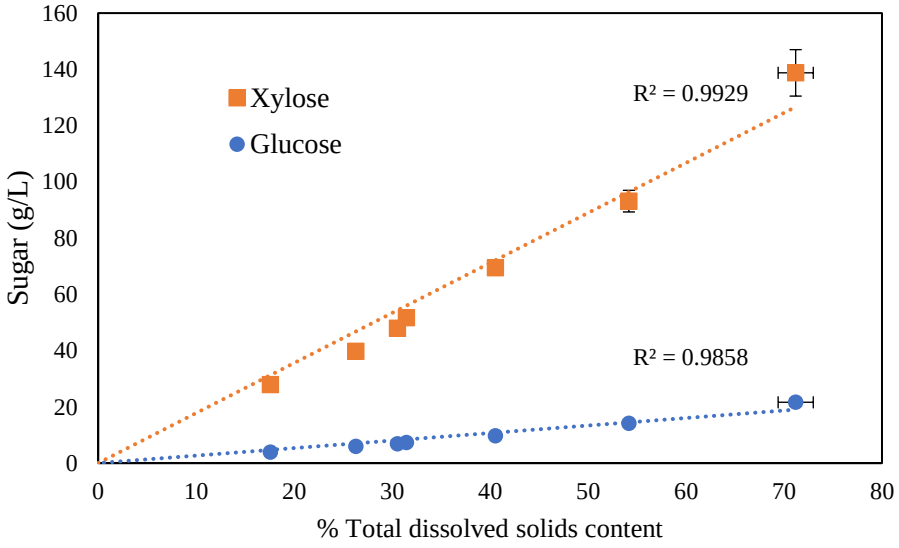

Figure 6: Relationship between TDS content and sugar concentrations across different stages in the multi-effect evaporator as represented in Table 8

From Figure 6 it is clear that the ratio between TDS content and both xylose and glucose remain constant across the first six streams (from Mg-5 to Mg-1A). This linear relationship between sugars and TDS indicates that both xylose and glucose is concentrated in the evaporator cascade without noticeable losses to degradation, unlike the case in the studies by Marques et al. (2009) and Novy et al. (2013), where xylose was converted into furfural. Figure 7 displays the combined sugar content of the different SSL streams from industry to show if it has the required sugar capacity to reach 40 g/L ethanol, widely considered as the economically feasible product titre to justify distillation costs (Ogden et al., 1990).

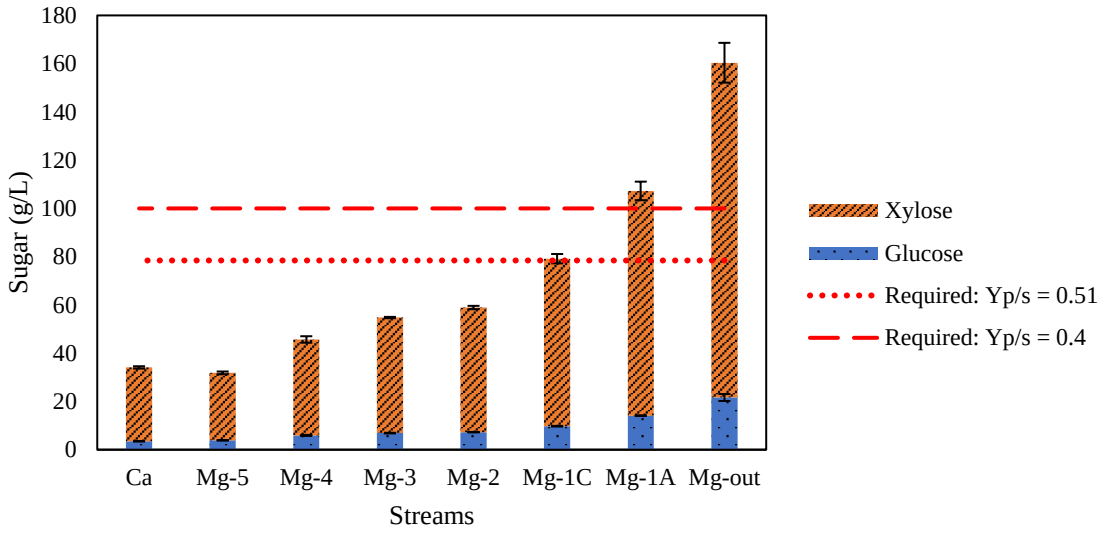

Figure 7: Total sugar contents of different SSL streams. The different required marks signify assumed product yield values

When assuming a maximum theoretical ethanol yield (Yp/s) of 0.51 g/g, a total sugar concentration of 78.4 g/L is required to achieve 40 g/L ethanol. From Figure 7 one can see that streams Mg-1C, Mg-1A and Mg-out are the only streams to meet the sugar requirement with total sugar concentrations of 79±1.9 g/L, 107±3.9 g/L and 160±8.4 g/L respectively. The assumption of 100% ethanol yield (i.e. 0.51 g/g) is however overly optimistic, since the maximum yield obtained from HW-SSL by *S. cerevisiae* is in the range of 0.4-0.43 (Pereira et al., 2013). When assuming an ethanol yield of 0.4 g/g, based on the maximum value obtained in previous studies (Table 2), only streams Mg-1A and Mg-out will be sufficient in achieving the required ethanol titre. Fermentation of Mg-out on the other hand would pose practical problems due to the high TDS content of this stream. High viscosity fermentations, caused by the high solid content, are challenging to operate and hence stream Mg-out was eliminated from further consideration. Stream Mg-1A is therefore the most suitable stream for ethanol production based on sugar composition and practical limitations. The composition of the only remaining stream, Mg-1A, stream is also similar to the high TDS SSL used by Brandt (2019).

4.1.2 Inhibitor content

The SSL streams contain compounds that are inhibitory to microorganism functions. Determining the concentrations of these compounds across different evaporator stages provide insight into the removal or concentration of these inhibitors, thus indicating whether high TDS or low TDS streams would be more fermentable based on toxicity levels. Figure 8 combines the main inhibitors from Table 8 with the respective TDS values for streams Mg-5 to Mg-1A to illustrate the effect of evaporation on the inhibitor concentration of different SSL streams.

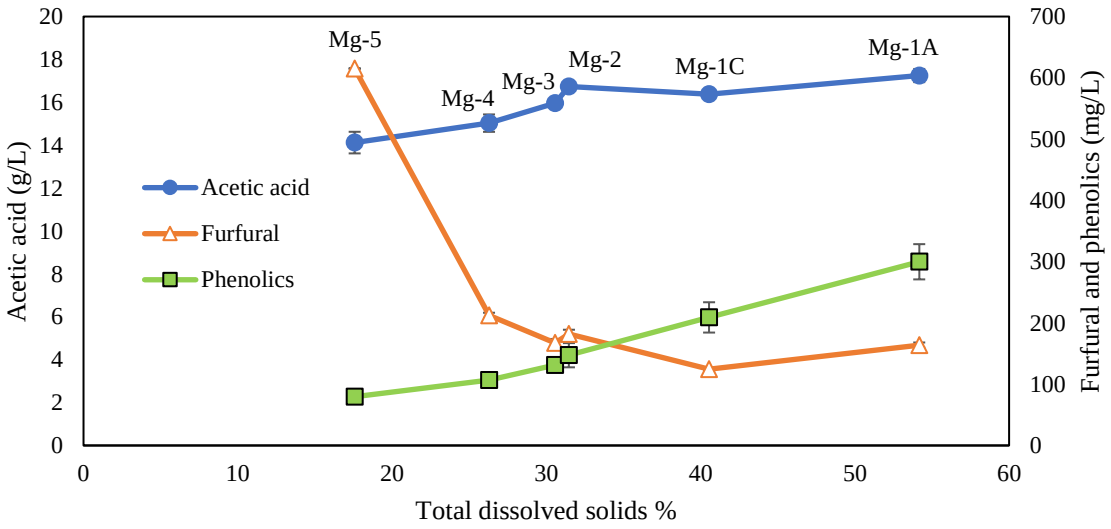

Figure 8: Inhibitor concentrations of streams Mg-5 to Mg-1A in the multistage evaporator.

Acetic acid and furfural concentrations of different streams indicate that large amounts of these components are removed from the SSL across the evaporator due to the volatile nature of these compounds. Phenolic compounds showed an increase corresponding to an increase in TDS, indicating that these components are not removed during evaporation.

Despite a 3-fold increase in TDS content from stream Mg-5 to Mg-1A, the acetic acid concentration is increased only from 14 g/L to 17 g/L. This is an indication that more than 60% of the acetic acid is removed in the evaporator, as was seen in the studies by Chipeta et al. (2005; Lawford and Rousseau, (1993). The acetic acid concentrations of the different streams are in the range of 14.1-17.2 g/L, which is higher than the 5 g/L deemed lethal in Section 2.1.4. It is therefore to be expected that acetic acid inhibition is a severe threat to the fermentations of this specific medium, regardless of which stream is fermented. The evaporator also appeared to remove more than 90% of the furfural from the SSL from stream Mg-5 to Mg-1A, as the

concentration is decreased by 60% after the first evaporation step and then stabilises at values of 124.5-212.5 mg/L despite further increase in TDS. All of the streams are however well below the 1 g/L furfural deemed inhibitory (Pereira et al., 2013; Taherzadeh and Karimi, 2011). Alternatively, the total phenolic concentration (accumulative values from Table 8) is 3.4-4.1 times higher in stream Mg-1A compared to Mg-5, while the TDS value increased 3-fold, thus indicating that these compounds are not removed during evaporation. The upwards gradient of the phenolic curve in Figure 8 also indicates that every stream displays an increase in phenolic compound concentration. The concentration of SSL therefore has the trade-off of increased sugar concentration at the expense of increased phenolic concentration as confirmed by (Taherzadeh and Karimi, 2011).

It is however difficult to evaluate the severity of the phenolics inhibition at a concentration of 80-300 mg/L, as literature describes phenolics of being inhibitory at the wide concentration range of 0.1-2 g/L (Chandel et al., 2013; Li et al., 2017; Taherzadeh and Karimi, 2011). However, the high acetic acid concentration of 14-17 g/L, coupled with the wide range of phenolic compounds are likely to inhibit fermentation severely regardless of which stream is selected, since aliphatic acids and phenolic compounds act synergistically to impede microorganisms (Jonsson et al., 2013; Kim, 2018; Li et al., 2017). Since all streams are likely to be too toxic to ferment ‘as is’, the best option is to select the concentrated stream Mg-1A as it also contains the highest sugar content of the eligible streams (Figure 7). The Mg-1A SSL can then be diluted with water to decrease the inhibitor concentrations to lower values than the other SSL streams, while still maintaining sugar concentrations similar to those streams. This strategy can be employed to determine the lowest medium dilution that can be fermented and serve as a building block to ultimately develop a fed batch process capable of fermenting pure SSL.

4.2 Baseline Fermentation - Synthetic media

As a base case fermentation, the performance of strains CelluX™4 and TFA7, were assessed in synthetic medium without inhibitors to investigate the xylose- and co-fermentation capability of the microorganisms. The mixture consisted of xylose and glucose at concentrations 30 g/L and 5.4 g/L respectively, representing the sugar composition of 40% Mg-1A SSL. Figure 9 and Table 9 display the fermentation profiles and kinetic parameters of the strains in this medium. A main hurdle in HW-SSL fermentations by recombinant *S. cerevisiae* strains is the inability to consume xylose efficiently with glucose as the preferred carbon source. To assess the ability of the strains to co-ferment glucose and xylose, the fermentations were divided into a glucose phase (GX-phase) and xylose-only phase (X-phase). The GX-phase extended from the start of the culture until the point of glucose depletion, after which the X-phase extended from the point of glucose depletion until the end of the fermentation. Figure 10 displays the performance of CelluX™4 and TFA7 during the different sugar phases.

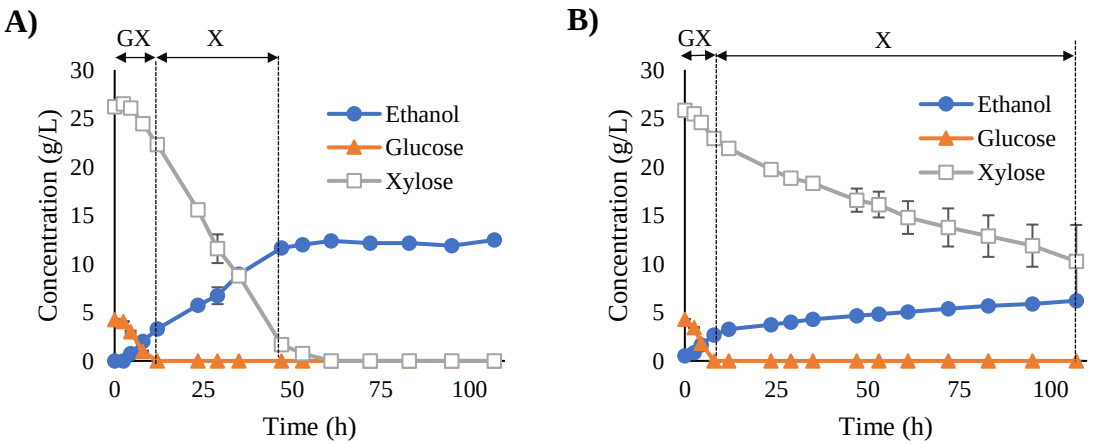

Figure 9: Fermentation profiles of CelluX™4 (**A**) and TFA7 (**B**) in synthetic medium. Horizontal arrows indicate the different sugar phases of the fermentations

Table 9: Kinetic parameters obtained in fermentation of synthetic medium[1]

Strain	$Ethanol_{max}$[2] (g/L)	$Y_{P/S}$[3] (g/g)	Productivity (g/L·h)	Xylose consumption rate (g/L·h)	Glucose consumption rate (g/L·h)
CelluX™4	11.7 ± 0.2	0.40 ± 0.01	0.248 ± 0.004	0.522 ± 0.008	0.356 ± 0.006
TFA7	5.7 ± 0.1	0.30 ± 0.02	0.058 ± 0.001	0.145 ± 0.035	0.535 ± 0.002

[1] ± symbols indicate the range of standard deviation
[2] The maximum ethanol concentration obtained in the fermentation
[3] Ethanol produced based on the total sugar consumed

CelluX™4 outperformed TFA7 in ethanol titre, yield and productivity by 100%, 30% and 330% respectively. One of the main factors in the superior performance of CelluX™4 is the higher ability to efficiently utilise xylose. CelluX™4 obtained a 260% higher xylose consumption rate than TFA7, which allowed it to deplete the xylose, while TFA7 only utilised 60% of this sugar. Brandt (2019) has remarked that the process of increasing robustness in these strains appear to have reduced the xylose capability.

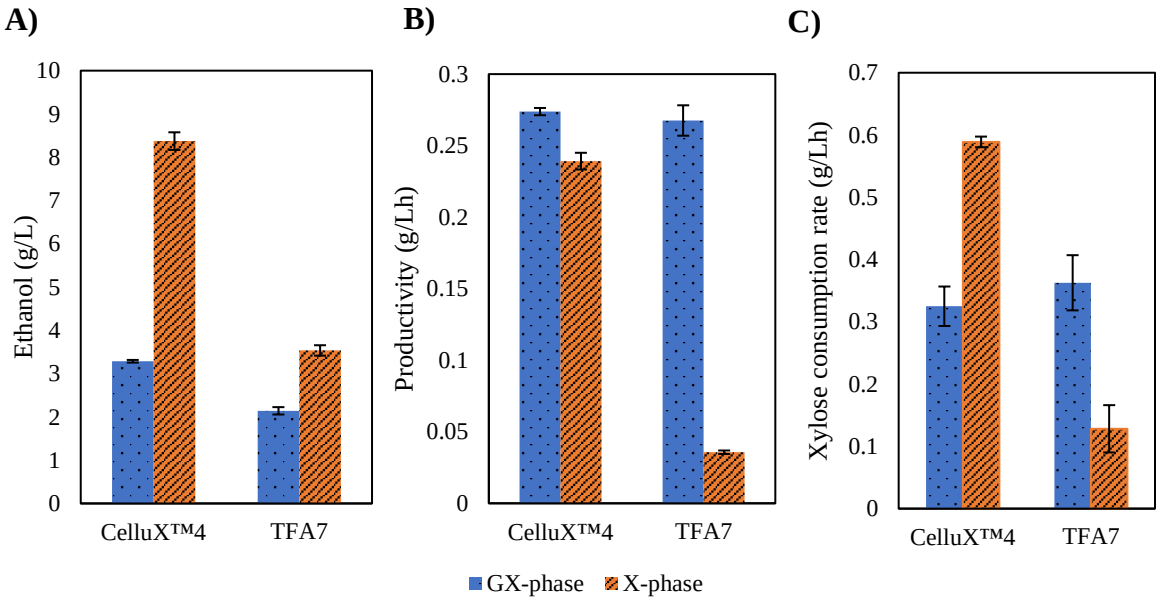

Figure 10: Ethanol titre (**A**)**,** Productivity (**B**) and xylose consumption rate (**C**) during different sugar phases in the fermentation of synthetic medium

CelluX™4 obtained a high productivity of 0.24-0.27 g/L·h throughout both the GX-phase and X-phase, indicating that the ethanol productivity during glucose-xylose co-fermentation could be maintained after glucose was depleted. The xylose consumption rate during the GX-phase was almost 50% lower than that obtained during the X-phase, since competition between glucose and xylose for transporters into the cells causes the xylose uptake to suffer (Kuyper et al., 2005; Moysés et al., 2016; Subtil and Boles, 2012)**.** Although glucose suppresses the xylose consumption rate in CelluX™4, co-fermentation of glucose and xylose still occurs as 15% of the xylose was consumed in the GX-phase. Additionally, the increased xylose consumption rate and sustained productivity during the X-phase compared to the GX-phase indicated that CelluX™4 did not lose the ability to utilise xylose as carbon source over the course of the fermentation. A flattening of the xylose curve before depletion of this sugar indicates severe metabolic stress, which is caused by the depletion of intercellular metabolites and low energy generation (Bergdahl et al., 2012; Matsushika et al., 2013; Novy et al., 2013). This metabolic

exhaustion often occurs in xylose capable recombinant *S. cerevisiae* strains and is either caused by too slow xylose consumption rates, which leads to the depletion of metabolites in cells thus initiating a carbon starvation state (Wei et al., 2018)(Matsushika et al., 2014; Moysés et al., 2016). The ability of CelluX™4 to continually utilise xylose at a constant rate until depleted, indicates that this strain is stable enough under xylose-only conditions to maintain metabolic processes, which is essential for successful fermentation of a xylose rich medium such as HW-SSL.

TFA7 on the other hand experiences an almost 90% decrease in volumetric ethanol productivity during the X-phase compared to the GX-phase indicating that the xylose consumption rate is much lower than that of glucose. The xylose consumption rate of strain TFA7 decreased by 50% during the X-phase compared to the GX-phase, implying that the presence of glucose leads to an increase in xylose capability for TFA7, which can either be caused by increased xylose transport or increased energy levels due to glucose. The low concentration of glucose can stimulate transporters with a higher affinity to xylose to assimilate it faster by 'pulling it along' with glucose as is observed her (Lane et al., 2018; Meinander and Hahn-Hägerdal, 1997; Souto-Maior et al., 2009). Alternatively, the energy demand of strains are sometimes not met during xylose-only fermentation, meaning that the supplication of low concentrations of glucose can assist in maintaining sufficient co-factor, metabolite and energy levels in cells (Bellissimi et al., 2009). Since no metabolome analysis were carried out on the cultures, it is not known whether the mechanism of xylose consumption rate improvement was related to the transport process or the increase in intercellular metabolite levels. Irrespective of the limiting mechanism in the X-phase, TFA7 experiences a decrease in xylose consumption rate and subsequently productivity, which further cause cells to deplete intercellular energy and maintenance sources indicating a carbon starvation state (Bergdahl et al., 2012; Matsushika et al., 2014; Wei et al., 2018). TFA7 therefore experiences metabolic exhaustion due to inefficient xylose fermentation abilities even in the absence of inhibitors, indicating that this strain will likely display inefficient xylose consumption in HW-SSL after glucose is depleted.

4.3 Batch fermentation in SSL

Batch fermentations were done in shake-flasks using Mg-1A SSL, which was chemically described in Table 8, in order to compare the performance of strains CelluX™4, TP1 and TFA7. Fermentations were conducted at three different SSL concentrations (20%, 40%, 60% (v/v)) in which Mg-1A SSL was diluted with R/O water, without adding extra sugar to the medium, in order to assess the impact of changes in sugar and inhibitor concentrations. The dilution with water is necessary since pure SSL, regardless of evaporation stage and TDS value, contains 14-17 g/L acetic acid, which is likely too toxic to allow efficient xylose fermentation as illustrated in Figure 2. Table 10 and Figure 11 display the kinetic parameters and fermentation profiles for all these experiments.

Table 10: Ethanol production parameters obtained in the fermentation of 20%, 40% and 60% (v/v) SSL fermentation by strains CelluX™4, TP1 and TFA7 [1]

SSL dilution/ Strain	Xylose consumption (%)	$EtOH_{max}$ [2] (g/L)	$Y_{P/S}$ [3] (g/g)	EtOH Productivity (g/L·h)	Xylose consumption rate (g/L·h)
20% SSL					
CelluX™4	74.1 ± 9.8	4.17 ± 0.64	0.31 ± 0.01	0.061 ± 0.009	0.170 ± 0.023
TP1	67.7 ± 5.1	2.79 ± 0.27	0.26 ± 0.02	0.024 ± 0.002	0.086 ± 0.012
TFA7	84.6 ± 12.3	3.47 ± 0.20	0.27 ± 0.03	0.030 ± 0.002	0.111 ± 0.016
40% SSL					
CelluX™4	80.7 ± 3.3	7.66 ± 0.57	0.32 ± 0.02	0.035 ± 0.003	0.099 ± 0.017
TP1	52.2 ± 2.3	4.91 ± 0.06	0.27 ± 0.01	0.022 ± 0.000	0.067 ± 0.005
TFA7	58.6 ± 2.1	5.54 ± 0.68	0.27 ± 0.02	0.025 ± 0.003	0.075 ± 0.005
60% SSL					
CelluX™4	21.9 ± 1.9	4.09 ± 0.04	0.24 ± 0.01	0.019 ± 0.000	0.041 ± 0.004
TP1	12.5 ± 3.8	2.74 ± 0.23	0.19 ± 0.02	0.019 ± 0.002	0.037 ± 0.011
TFA7	24.3 ± 2.9	4.89 ± 0.35	0.25 ± 0.01	0.025 ± 0.002	0.053 ± 0.007

[1] ± symbols indicate the range of standard deviation
[2] The maximum ethanol concentration obtained in the fermentation
[3] Ethanol yield on total sugar consumed

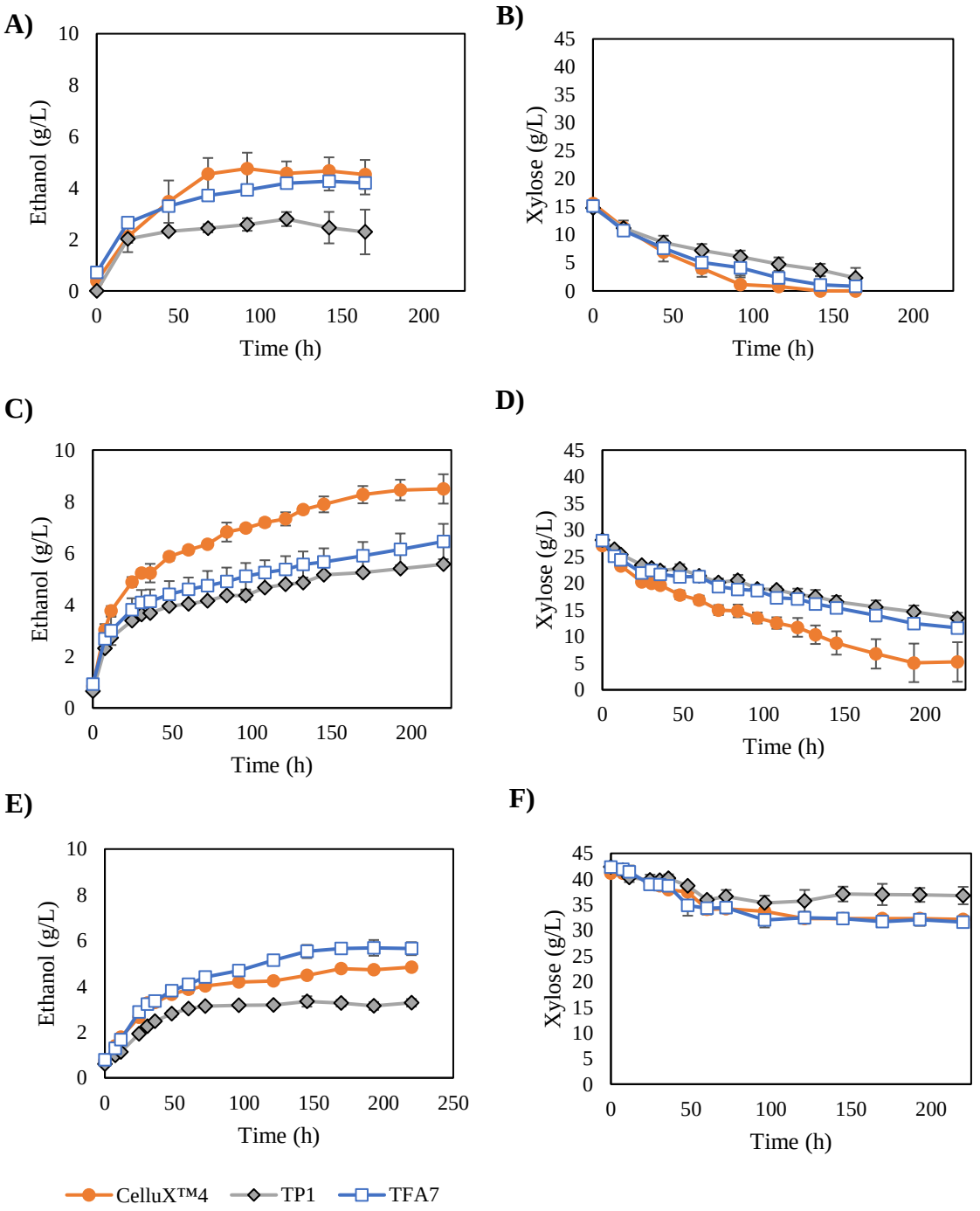

Figure 11: Fermentation profiles of different yeast strains at 20% SSL (**A**, **B**), 40% SSL (**C**, **D**), and 60% SSL (**E**, **F**)

CelluX™4 is the best performing strain in 20% and 40% SSL fermentations, as it outperformed the other two strains in terms of ethanol titre as seen in Figure 11. At 20% SSL medium, CelluX™4 obtained an ethanol titre of 4.2±0.6 g/L compared to 2.8±0.3 g/L and 3.5±0.2 g/L of TP1 and TFA7, respectively (Table 10). At 40% SSL, CelluX™4 obtained 7.7±0.6 g/L which is 60% and 40% higher than that of TP1 and TFA7, respectively. The superior ability of CelluX™4 to produce ethanol at 40% SSL is two-fold, since this strain obtained an ethanol yield of 0.32 g/g compared to that of 0.27 g/g of the other two strains, and CelluX™4 also utilised 80% of the available xylose, compared to 52% and 59% of TP1 and TFA7, respectively. As was seen in the synthetic medium, CelluX™4 has superior xylose utilising abilities to TFA7, which in turn leads to higher ethanol titres and/or volumetric productivity.

However, at 60% SSL fermentations the strain TFA7 was more capable of tolerating the high inhibitor concentrations than the other strains as it performed best in terms of ethanol concentration and productivity as seen in Figure 11. TFA7 obtained an ethanol titre of 4.9±0.4 g/L which is 20% and 80% more than the titres obtained by CelluX™4 and TP1, respectively (Table 10). TFA7 also achieved a volumetric ethanol productivity of 0.025 g/L·h during the fermentation of 60% SSL medium, which is 30% higher than that of the other strains. The intricate nature of yeast metabolism and lignocellulosic medium causes strains to vary performance based on fermentation medium or mode, as strains react differently to inhibitors (Cunha, Romaní, et al., 2019; Modig et al., 2008). This is also the case with CelluX™4 which showed dominant xylose utilisation abilities in synthetic media, 20% and 40% SSL, but was severely impeded at 60%. TFA7 then outperformed CelluX™4 at 60% SSL, proving that it is more capable of tolerating high inhibitor concentrations.

At an SSL dilution of 60%, the accumulative inhibition effects reach a lethal limit for the strains CelluX™4 and TP1 as the ethanol titre and yield of these recombinant strains are negatively affected at the inhibitor levels present at this SSL dilution, compared to 40% SSL. All three strains achieved their highest ethanol titre at 40% SSL with CelluX™4, TP1 and TFA7 achieving values of 7.7±0.6 g/L, 4.9±0.1 g/L and 5.5±0.7 g/L, respectively (Table 10). CelluX™4 and TP1 showed a 50% and 40% decrease in ethanol titre at 60% SSL compared to 40% SSL, indicating that 40% SSL serves as a turning point whereafter inhibition levels become severe for these two strains. Similarly, CelluX™4 and TP1 showed a decrease of 20% and 30% respectively in ethanol yield at 60% SSL compared to the values obtained in 20-40% SSL, while TFA7 showed no significant difference at 60% SSL and obtained a value of 0.25-0.27 g/g across all dilutions. The microbial inhibitors in SSL has been known to affect the

ethanol productivity of cells, without affecting their yield, as was seen for all three strains at 40% SSL compared to 20% SSL (Kim, 2018; Taherzadeh and Karimi, 2011). However, at sufficiently high inhibitor concentrations, yield can also be reduced, indicating irreversible cell damage (Taherzadeh and Karimi, 2011; Zhang et al., 2014) as likely occurred for CelluX™4 and TP1 at 60%. For the system at hand, 60% SSL can be viewed as the lethal inhibitor limit as it severely impedes two of the three yeast strains, while also preventing the third from improving on product titre obtained at 40% SSL. Unlike the other two strains, TFA7 is less affected at high inhibitor concentration in terms of ethanol yield and titre, due to the overexpression of robustness genes (TAL1, ARI1 and ADH6) (Brandt, 2019), to increase tolerance to furfural and acetic acid which are both present in SSL. It is surprising that TP1 was more affected than TFA7 at the high inhibitor levels at 60% SSL, since this strain underwent one more round of gene overexpression (PAD1) to improve tolerance to phenolic compounds than TFA7 (Brandt, 2019).

Xylose consumption rate and associated ethanol productivity is more sensitive to inhibition than ethanol yield, as markable decreases are evident in these parameters at 40% SSL compared to 20% SSL fermentation. CelluX™4, TP1 and TFA7 obtained xylose consumption rates of 0.17±0.02 g/L·h, 0.09±0.01 g/L·h and 0.11±0.02 g/L·h respectively at 20% SSL. A 20-50% reduction in xylose consumption rate occurred in 40% SSL compared to 20% SSL, while an additional reduction of 30-60% occurred in 60% SSL compared to 40% SSL. The ethanol productivity of CelluX™4 also decreased by 45% with every increase in SSL concentration compared to the value of 0.06 g/L·h obtained in 20% SSL. Inhibitors sometimes affect the productivity and sugar consumption rates without reducing the ethanol yield, thus stating that productivity is more sensitive to inhibitors than ethanol yield (Kim, 2018; Taherzadeh and Karimi, 2011). Although 40% SSL delivered the highest ethanol concentration due to the trade-off between inhibitor and sugar concentrations, the toxicity at this dilution already affected the xylose fermentation rate negatively. When fed batch strategy is applied, the initial SSL percentage should be as low as allowable, since this will increase the xylose fermentation rate of the culture and subsequently increase productivity.

Acetic acid is one of the main inhibitors in HW-SSL as it is often present at the highest concentration of all the toxic compounds as seen in Figure 8 and literature (Jonsson et al., 2013; Ko et al., 2020; Pereira et al., 2013). Detoxification of acetic acid is therefore important as it can serve as an indicator of the robustness of yeast strains. Detoxification of acetic acid is

evaluated at 20% and 40% SSL fermentations for all three strains at the time of maximum ethanol concentration. Figure 12 display the acetic acid removed.

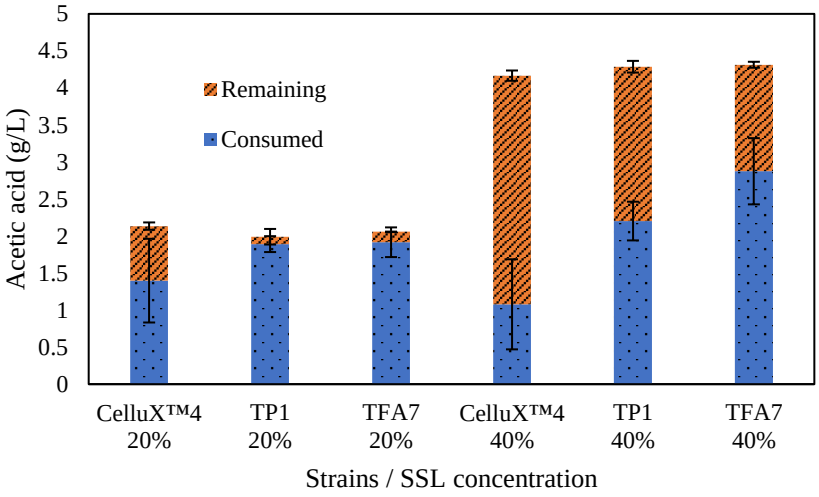

Figure 12: Acetic acid detoxification in 20% and 40% SSL fermentations

TP1 and TFA7 showed a higher ability to consume acetic acid than CelluX™4, which is likely due to the rational engineering of the two laboratory strains, aimed at increasing tolerance towards weak acids (Brandt, 2019). Strains TP1 and TFA7 nearly depleted the acetic acid in the 20% SSL fermentations (Figure 12), while CelluX™4 showed more variance by consuming 65±25% of the 2 g/L. At 40% SSL fermentation, CelluX™4, TP1 and TFA7 consumed 1.1±0.6 g/L, 2.2±0.3 g/L and 2.9±0.4 g/L acetic acid respectively, again indicating that the two latter strains have a superior ability to remove this inhibitor from the fermentation due to the overexpression of genes to increase tolerance towards weak acids. TP1 and TFA7 underwent a sequence of rational engineering steps to increase tolerance to weak acids and furfural, with TP1 also harbouring overexpressed genes to additionally enable robustness against organic solvents and phenolic compounds (Brandt, 2019)

4.4 Sensitivity of xylose capability in *S. cerevisiae* strains in HW-SSL fermentations

In Section 4.3 most fermentations showed a slowdown and pre-mature stop in ethanol production before the xylose could be depleted. This incomplete utilisation of xylose is a limitation to the ethanol titre in the fermentation of HW-SSL. The fermentation of xylose puts recombinant *S. cerevisiae* strains under metabolic stress as the low consumption rate causes the depletion of metabolites in cells such as cofactors and energy sources (Bergdahl et al., 2012; Matsushika et al., 2013, 2014). In Section 4.2, however, it was proved that CelluX™4 is a highly efficient xylose capable strain, as it depleted xylose in synthetic media without showing any slowdown in metabolism as measured by xylose consumption rate. Xylose metabolism is generally more easily negatively affected by inhibitors, as the additional stress and energy demand of inhibitor resistance can lead to the depletion of metabolites such as nicotinamide adenine dinucleotide phosphate NAD(P)H and adenosine triphosphate (ATP) (Moysés et al., 2016; Wang et al., 2014).

The hypothesis is therefore that the xylose capable strain, CelluX™4, loses its ability to utilise xylose due to the additional stress placed on the cells by inhibitors in the SSL, causing cells to deplete internal metabolites and lose viability leading to carbon starvation. Therefore, it is expected that glucose would be consumed despite the high toxicity of SSL. To determine whether this metabolic exhaustion occurs specifically in the fermentation of xylose, and is not caused by other unknown factors which would occur in glucose fermentation as well, a 60% SSL batch fermentation was compared to a medium where 20 g/L glucose was added to 60% SSL. Figure 13 displays the fermentation profiles obtained by CelluX™4 in 60% SSL medium and 60% SSL medium supplemented with glucose.

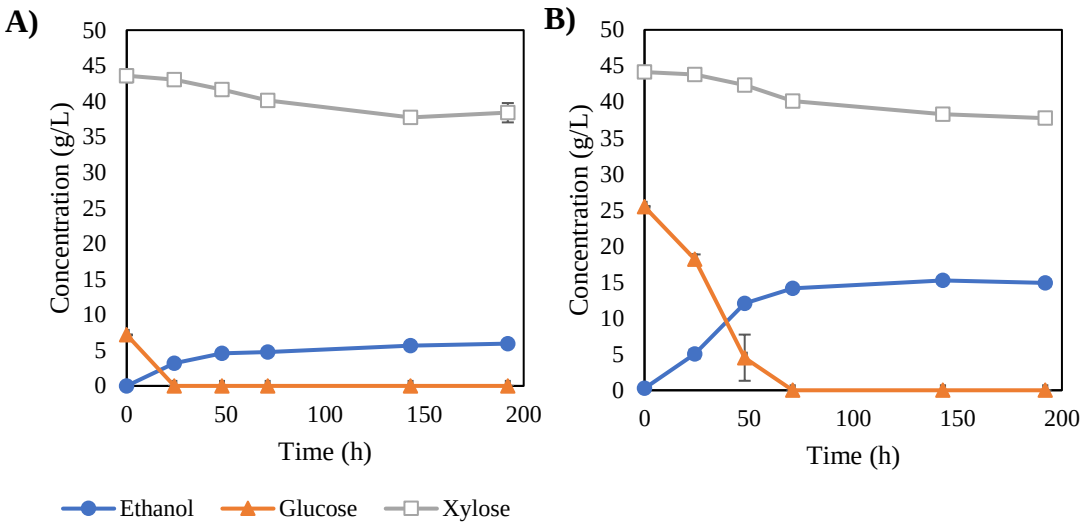

Figure 13: Fermentation profiles in batch cultures of 60% SSL (A) and 60% SSL supplemented with 20 g/L glucose (B) by CelluX™4

The low ethanol titre seen in 60% SSL is directly caused by cells losing the ability to utilise xylose as carbon source, due to the lethal effect of inhibitors on the consumption path. The culture where 20 g/L glucose was added to the medium, obtained 14.9 g/L ethanol, which is 150% higher than that of normal 60% SSL medium, since the former culture depleted 25 g/L glucose, compared to the 7 g/L in 60% SSL. Neither culture was however able to utilise xylose effectively as only 12-13% of the available xylose was utilised. CelluX™4 is therefore capable of consuming glucose at the high inhibitor levels of 60% SSL, although the xylose utilisation is minimal, indicating that even this highly xylose-capable strain experiences carbon starvation during xylose fermentation at sufficiently high inhibitor levels.

4.5 Fed batch fermentations

The results in Section 4.3 indicated that xylose consumption and in effect ethanol production will end before the xylose has been depleted if the lethal inhibitor concentration is exceeded in the culture. At 60% SSL the yeast cells experienced severe stress brought on by microbial inhibitors which caused a decrease in xylose consumption, ultimately leading to metabolic exhaustion. Fed batch culture will provide an opportunity to gradually expose the yeast cells to inhibitors, instead of 'shocking' the culture with a too toxic medium. By feeding the inhibitors at the rate which the yeast cells can detoxify them, one can keep the inhibitor concentration below the lethal limit.

4.5.1 Shake flask pulse-fed fermentations

Instead of adding all the high TDS Mg-1A SSL upfront in a 60% SSL batch fermentation, the SSL was added in pulses to reduce the inhibitor concentrations and associated metabolic stress responses of the yeast cells, in an attempt to increase ethanol production. Two-pulse and four-pulse feeding schemes were conducted, to resemble fast feeding and slow feeding respectively, according to Table 11 to increase the SSL concentration from 20% to 60%. Table 12 and Figure 14 respectively display the fermentation parameters and profiles of the batch and fed batch cultures.

Table 11: Different pulse feeding schemes for fed batch fermentation of SSL

	Two-pulse feeding scheme		**Four-pulse feeding scheme**	
Time (h)	**Pulse volume (ml)**	**Cumulative % SSL (v/v)**	**Pulse volume (ml)**	**Cumulative % SSL (v/v)**
0	0	20	0	20
48	24	44	12	34
72	0	44	12	44
96[1]	24	60	12	52
120	0	60	12	60

[1]This timestep signifies the end of the first half of the feeding regime (feeding midpoint) which is mentioned in upcoming graphs

Table 12: Ethanol production parameters obtained in 60% SSL batch and fed batch fermentations

Strain	Fermentation mode	$EtOH_{max}$ (g/L)	Productivity (g/L·h)	$Y_{P/S}$ (g/g)
CelluX™4	Batch	4.09 ± 0.04	0.019 ± 0.000	0.24 ± 0.01
	Two-pulse feeding	7.75 ± 0.06	0.054 ± 0.00	0.26 ± 0.01
	Four-pulse feeding	7.85 ± 0.03	0.051 ± 0.00	0.23 ± 0.01
TFA7	Batch	4.89 ± 0.35	0.025 ± 0.002	0.25 ± 0.01
	Two-pulse feeding	7.18 ± 0.21	0.040 ± 0.00	0.33 ± 0.03
	Four-pulse feeding	7.42 ± 0.62	0.041 ± 0.003	0.31 ± 0.06

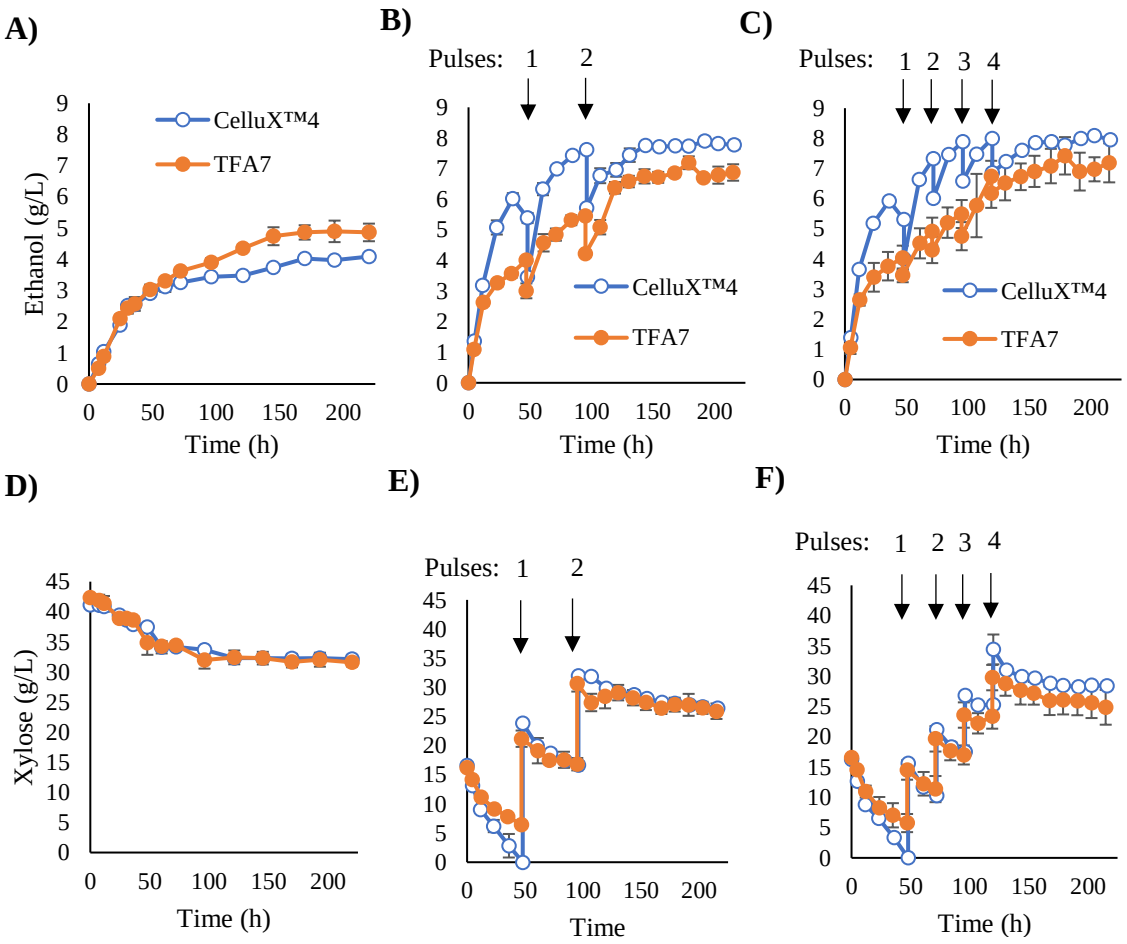

Figure 14: Fermentation profiles of 60% SSL for batch fermentation (**A**; **D**), fed batch configuration for two-pulse (**B**; **E**) and four-pulse (**C; F).** The arrows represent the times at which SSL pulses were administered.

There was no significant difference in final ethanol titres between the two-pulse and four-pulse feeding schemes for either strain, as CelluX™4 and TFA7 obtained 7.8-7.9 g/L and 7.2-7.4 g/L respectively (Table 14). It is likely that both feeding schemes were too similar for any notable improvement to occur with the four-pulse scheme compared to the two-pulse scheme.

Both strains did display an improvement in pulse fed batch fermentations compared to simple batch cultures. CelluX™4 and TFA7 displayed improvements of 90% and 50% respectively in terms of ethanol titre in pulsed fed batch compared to batch fermentations (Table 14). Furthermore, feeding resulted in a 190% and 60% increase in volumetric ethanol productivity for CelluX™4 and TFA7, respectively. By introducing the inhibitors via multiple pulses, the yeast cells are allowed to operate below the lethal inhibitor concentration, thus improving ethanol titre and productivity (Modig et al., 2008; Nilsson et al., 2001; Zhang et al., 2014).

In order to investigate the influence of increasing SSL concentration on fermentation performance, the fermentations displayed in Figure 14 were divided into different phases. The changes throughout the fermentation were assessed by calculating and comparing fermentation parameters between pulses. Figure 15 display these parameters for the batch phase (0-48h), first feeding half (48-96h) and second feeding half (96-max ethanol).

CelluX™4 is the superior strain in terms of fermentation rate, enabling it to outperform TFA7 over the whole course of the fermentation (Figure 15). Although the final ethanol titre of the two strains is similar with CelluX™4 and TFA7 achieving 7.8 ± 0.1 g/L and 7.2 ± 0.2 g/L, respectively, CelluX™4 was superior in fermentation rate since it achieved a productivity 35% higher than that of TFA7 (Table 12). The high xylose consumption rate and subsequent productivity enabled CelluX™4 to achieve 50% and 70% higher product titres than TFA7 over the batch phase and first feeding half phase, respectively (Figure 15-D). CelluX™4 was also seen to achieve the highest ethanol titre and productivity in the 40% SSL batch culture in Section 4.3.

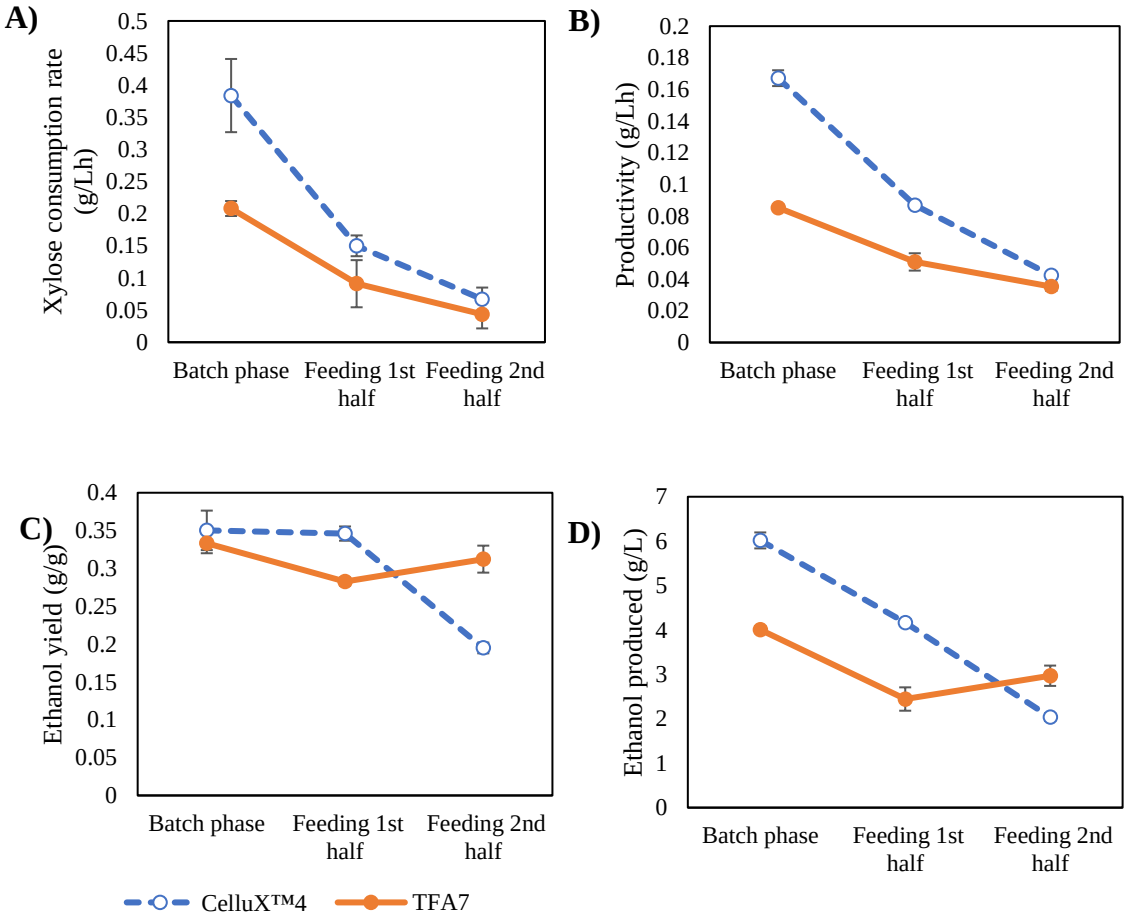

Figure 15: Xylose consumption rate (**A**), productivity (**B**), ethanol yield (**C**) and titre increases (**D**) for different phases in the pulse fed batch fermentation

The robust nature of TFA7 allowed it to catch up to CelluX™4 towards the end of the fermentation. Despite an initial lag behind CelluX™4 during batch phase and first feeding half phase of the pulse fed batch fermentation, the two strains obtained similar productivity values of 0.04 g/L·h during the second feeding half (Figure 15-B). This change is brought on by the ability of TFA7 to sustain its ethanol yield of 0.31±0.02 g/g at the last fermentation phase, compared to the yield of CelluX™4 of 0.19±0.01 g/g; a 40% decrease relative to the yield of the previous fermentation phase (Figure 15-C). The superior yield of TFA7 also allowed it to produce 25% more ethanol than CelluX™4 after the final SSL pulse (Figure 15-D). This ability of TFA7 to outperform CelluX™4 at 60% SSL was also observed in the batch cultures of Section 4.3, confirming the high robustness of TFA7. Although TFA7 is not suitable for the fermentation of 20-40% SSL due to the low xylose affinity, likely caused by the rational engineering techniques employed to increase inhibitor tolerance (Brandt, 2019), the resulting robustness makes the strain more suitable for 60% SSL than CelluX™4.

4.5.2 Fed batch fermentations with continuous feeding in a 5L bioreactor

Strain performance was assessed at different volumetric feed-rates and inoculum sizes in fed-batch cultures with continuous feeding. By adding SSL gradually to a culture, as opposed to large pulses, it is more probable to condition the yeast strains to HW-SSL and prevent subjecting the cells to lethal shocks of inhibitors. The effect of gradual substrate addition was tested in a 5-L bioreactor. All fed batch fermentations were initiated with a starting volume of 2L, consisting of 20% SSL and 80% a mixture of R/O water and inoculum liquid. Two volumetric feeding profiles were tested in which the volume was increased to 4.6 L in 6 days (13-30 mL/h) and 9 days (8-15 mL/h) respectively by the addition of pure high TDS SSL from the second last evaporator stage (Mg-1A), to obtain a final SSL concentration of 65% (Figure 16). The projected feeding rates of the peristaltic pumps were verified by marking and measuring the fed volume at every sampling point. Batch fermentations were initiated at the final working volume and SSL concentration of 4.6 L and 65% as benchmark to assess the advantage of fed batch. SSL feeding rates and subsequent increase in SSL concentration with time is displayed in Figure 16.

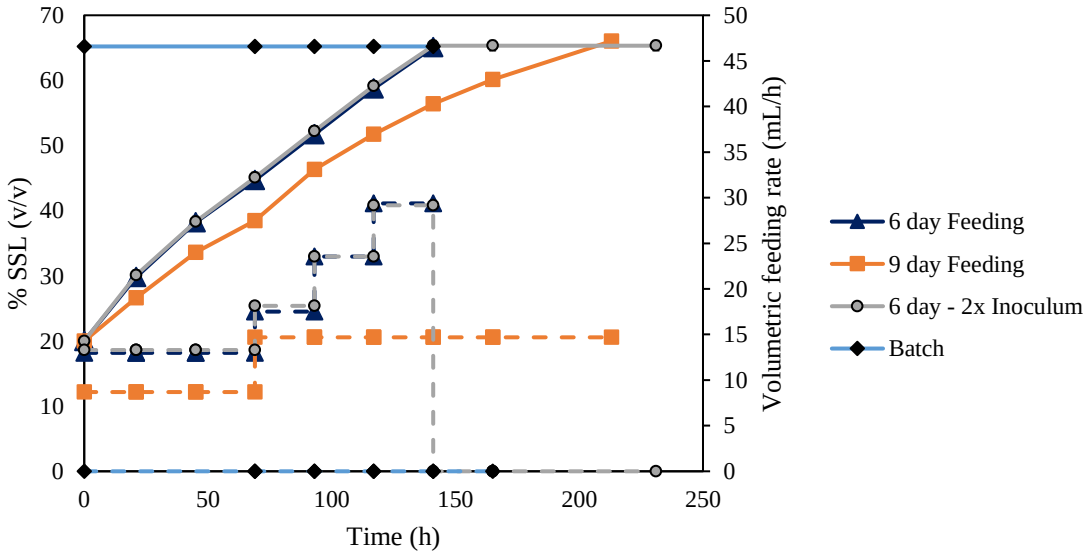

Figure 16: Change in SSL loading (solid lines) and volumetric feeding rate (dotted lines) over time in fed batch bioreactor fermentations compared to simple batch cultures

The effect of cell density was assessed by repeating the 6-day feeding scheme by doubling the inoculum size from 300 ml to 600 ml. This increased the yeast cell concentration from 0.4 g/L to 0.8 g/L (based on final working volume). All cultures were conducted in duplicate. Decreased flowrate and increased cell density are well-documented strategies for increasing ethanol production in lignocellulosic fermentations such as HW-SSL (Taherzadeh and Karimi,

2011; Zhang et al., 2014). The effect of a slower feeding profile and larger inoculum size are respectively assessed in this section for HW-SSL fed batch fermentations. Figure 17 and Table 13 and display the ethanol profiles and fermentation parameters of the bioreactor cultures, respectively. Figure 18 displays the sugar and acetic acid consumption during the bioreactor runs.

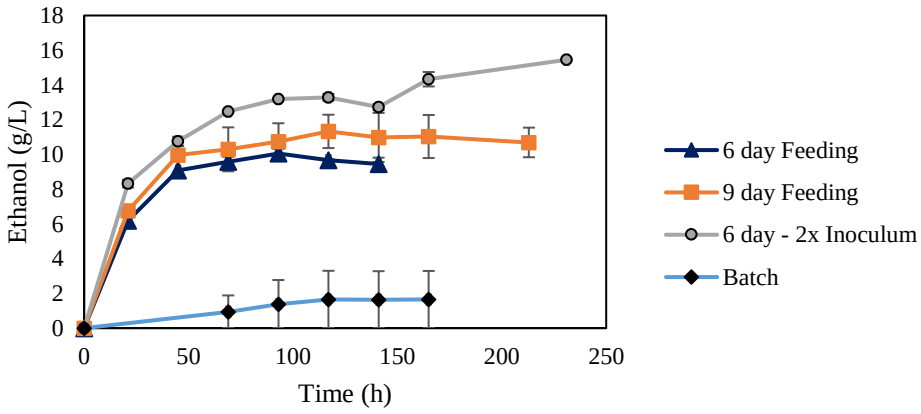

Figure 17: Ethanol production profiles over time of batch and fed batch fermentations in 5-L bioreactors

Table 13: Ethanol production parameters in 5-L bioreactor batch and fed batch fermentations

Filling period (days)	**Initial biomass (g/L)**	**$EtOH_{max}$ (g/L)**	**$Y_{P/S}$[1] (g/g)**	**EtOH Productivity (g/L·h)**	**Xylose consumption rate (g/L·h)**
Batch	0.4	1.64 ± 1.64	0.11 ± 0.11	0.014 ± 0.014	0.019 ± 0.011
6	0.4[2]	9.5 ± 0.4	0.37 ± 0.02	0.067 ± 0.003	0.130 ± 0.011
9	0.4[2]	10.7 ± 0.9	0.35 ± 0.02	0.050 ± 0.004	0.098 ± 0.009
6	**0.8[2]**	12.7 ± 0.2	0.43 ± 0.02	0.090 ± 0.001	0.149 ± 0.013

[1] Ethanol yield on total sugar consumed
[2] Calculated based on final working volume during fed batch fermentations

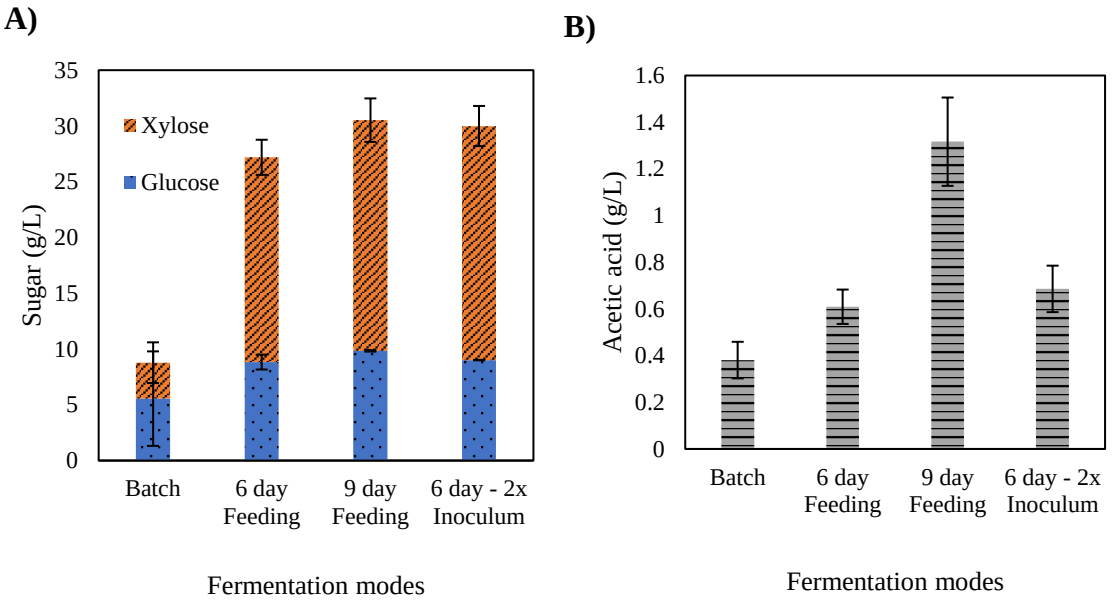

Figure 18: Sugar consumption **(A)** and acetic acid consumption **(B)** in bioreactor cultures

Batch fermentations were highly unstable and only obtained 1.6 g/L ethanol (Table 13). The 6-day feeding scheme outperformed the batch fermentation in terms of ethanol titre, yield and productivity by 500%, 70% and 400%, respectively, indicating that fed batch strategy decreases the metabolic stress on cultures and increases ethanol production. 65% SSL media contained 8.2 g/L acetic acid, which is substantially higher than the 5-6 g/L considered inhibitory to yeast strains and lethal to xylose fermentation as discussed in Section 2.1.4 (Giannattasio et al., 2013; Jönsson and Martín, 2016; Kim, 2018). By subjecting the yeast to the lethal inhibition from the beginning, as was done in the batch culture, the cells could not maintain their viability and metabolic exhaustion ensued.

By decreasing the feed rate of SSL, the ethanol titre and *in situ* detoxification ability of the culture was improved. By increasing the feeding time from 6 days to 9 days, a marginal improvement in ethanol titre occurred from 9.5±0.4 g/L to 10.7±0.9 g/L (Table 13). The product increase in the longer feeding time is likely caused by the fact that 7% more sugar was consumed during the 9-day fermentation compared to the 6-day fermentation (Figure 18-A). By feeding the SSL slower, the acetic acid consumption increased by 120% (Figure 18-B). Slower feeding allows more efficient *in situ* detoxification by cultures, as less stress is placed on the cells (Kim, 2018; Taherzadeh and Karimi, 2011; Zhang et al., 2014). Increasing the feeding time from 6 days to 9 days resulted in a 25% decrease in ethanol productivity. Han et al. (2017) has remarked that feeding rate selection can lead to a trade-off where slower feeding rates increase product titre at the expense of productivity.

Increasing the initial cell density from 0.4 g/L to 0.8 g/L proved an efficient strategy to increase fermentation performance. The 0.8 g/L dry weight fed batch culture outperformed the 0.4 g/L counterpart in terms of ethanol titre and productivity by 30% and 35% respectively, obtaining values of 12.7 g/L and 0.090 g/L·h. Surprisingly, doubling the inoculum resulted in only a 15% increase in xylose consumption rate, whereas one would expect an increase closer to 100%. The increased cell concentration did however increase the ethanol yield from 0.37 g/g to 0.43 g/g, indicating an increase in culture tolerance to inhibitors (Taherzadeh and Karimi, 2011; Zhang et al., 2014).

After feeding has stopped, almost 70% of xylose fed into the reactor still remained, indicating the potential to further increase the ethanol titre. Taherzadeh et al. (2000) observed that residual sugar can still be utilised for ethanol production after feeding of hydrolysates had stopped, despite the high inhibition at this point. The double inoculum 6-day feeding fermentation was continued for an additional 4 days after feeding stopped, increasing the final ethanol titre from 12.7 g/L to 15.5 g/L at the expense of a 25% decrease in productivity. This loss in ethanol productivity was also observed in the shake flask batch cultures and is caused by metabolic exhaustion during the fermentation of xylose, after glucose is depleted, in the presence of high inhibitor concentrations (Novy et al., 2013; Taherzadeh and Karimi, 2011; Wei et al., 2018). This extended fermentation phase does however indicate that CelluX™4 possess the ability to obtain higher ethanol titres than 12 g/L but is limited by the xylose consumption rate and fermentation time.

The usage of the novel recombinant *S. cerevisiae* strain CelluX™4 in combination with fed batch strategy obtained an ethanol titre that is among the highest reported in literature on non-detoxified HW-SSL. However, the work in this study applied strict industrially relevant conditions, with no SSL pretreatment or detoxification, thus straining the yeast cells more than previous reported literature. The fermentation conditions and parameters obtained in this study are displayed in Table 14 along with that of the highest performing studies, adopted from Table 2.

Table 14: Ethanol production from non-detoxified HW SSL

TDS	% SSL	Hexose (g/L)	Xylose (g/L)	Acetic acid (g/L)	Inoculum strength	pH	Nutrients[1] (g/L)	Species (strain)	Time (h)	EtOH (g/L)	Productivity (g/L·h)	$Y_{P/S}$ (g/g)	Ref
								S. cerevisiae					
35	65	9.1	64	8.7	0.8 g/L[2]	5	CSL - 5	CelluX™4	141	12.7	0.090	0.43	This study
35	**65**	**9.1**	**64**	**8.7**	**0.8 g/L[2]**	**5**	CSL - 5	**CelluX™4**	**231**	**15.5**[3]	**0.067**	**0.43**	**This study**
35	65	9.8	71	8.7	0.4 g/L[2]	5	CSL - 5	CelluX™4	213	10.7	0.050	0.35	This study
35	65	8.8	62	7.3	0.4 g/L[2]	5	CSL - 5	CelluX™4	141	9.5	0.067	0.37	This study
20	100	14.9	21.8	-	4 g/L	6.5	-	259ST	120	12	0.1	0.42	(Helle et al., 2004)
								S. stipitis					
-	60[4]	5	40	8.8	0.4 (OD)	5.5	YE – 2.5 DAP – 2 AS – 1 MSHH – 0.5	Isolate C_4	350	12.2	0.04	0.39	(Henriques et al., 2018)
								E. coli					
20-22	100	11	22.4	8.6	0.5 g/L	7	Tryptone – 10 YE – 5	ATCC 11303	120	11.2	0.16	0.4	(Lawford and Rousseau, 1993)

[1] Abbreviations are as follows: Corn steep liquor – CSL; Yeast extract - YE; $(NH_4)_2HPO_4$ – DAP; $(NH_4)_2SO_4$ – AS; $MgSO_4 \cdot 7H_20$ - MSHH
[2] Cell dry weight was calculated based on the final working volume in fed batch fermentations
[3] Concentration obtained after 6 day feeding scheme was continued in batch configuration for 4 more days after feeding was completed
[4] Pre-treatment included adjusting HW SSL to pH 7, aeration with compressed air after which lignosulphonates and other colloids were removed by means of centrifugation and microfiltration

An ethanol titre of 12.7 g/L was achieved during the 6-day feeding scheme devised in this study, utilising a low cell density of 0.8 g/L, placing it with the top results achieved by previous studies which achieved final concentrations of 11.2-12.2 g/L. An extended batch phase of 4 days, after feeding was stopped, allowed the fermentation to reach stationary phase and obtain 15.5 g/L ethanol, which is the highest titre achieved in non-detoxified HW-SSL to the author's knowledge.

Additionally, the TDS of the SSL in this study was 60% higher than that used in the studies of Helle et al. (2004) and Lawford and Rousseau, (1993), indicating that CelluX^{TM}4 might have been subjected to higher levels of osmotic stress and inhibition by inorganic salts than previous studies. It is also likely that the TDS content of the SSL used by Henriques et al. (2018) was lower than 35%, despite comparably high sugar concentrations, since pretreatment by adjustment to pH 7 and removal of colloids would have decreased the lignosulphonate and other lignin derivative concentrations (Chandel et al., 2012; Jonsson et al., 2013).

CelluX^{TM}4 was subjected to a final acetic acid concentration of 8.7 g/L, which compares to the 8.6-8.8 g/L of the other studies in Table 14. Helle et al. (2004) and Lawford and Rousseau, (1993) utilised pH values of 5.3-6.5 and 7, respectively, which reduces the inhibitory effect of weak organic acids such as acetic acid (Casey et al., 2010; Ko et al., 2020; Novy et al., 2013). Lawford and Rousseau, (1993) further utilised high concentrations of laboratory growth medium (10 g/L Tryptone, 5 g/L yeast extract) as nitrogen sources in the fermentation. Although this medium increases cell viability and mitigates inhibition, the chemicals are too expensive to use on industrial scale (Helle et al., 2008; Jørgensen, 2009). Henriques et al. (2018) mitigated the acetic acid inhibition by implementing a two-phase aeration process, in which the acetic acid was depleted in the first phase during aerobic fermentation. This also allowed the fermentation to develop a dense cell culture (6 g/L), which can in turn increase inhibitor tolerance (Cantarella et al., 2004; Chung and Lee, 1985; Pienkos and Zhang, 2009). Helle et al. (2004) used the same principle by initiating the fermentation with a cell concentration of 4 g/L, which is not economically desirable due to the cost of inoculum development and maintenance (Helle et al., 2008; Wingren et al., 2003).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

i. The usage of a low TDS stream holds no benefit for fermentation since the acetic acid level of all the SSL streams are too high (14-17 g/L) to allow fermentation of xylose. It is necessary to dilute a thick SSL stream with water to obtain sub-lethal inhibitor levels.

ii. CelluX™4 is the superior xylose capable strain compared to TFA7 and TP1, as evident in synthetic medium and low SSL concentration, but the ability to utilise xylose perishes at high SSL concentrations due to lethal inhibitor concentrations. TFA7 is the more robust strain as evident in acetic acid *in situ* detoxification ability (Figure 12) and superior performance in high SSL medium batch fermentations (Figure 11-**E**,**F**), but the fermentation rate is restricted by an inherent xylose utilisation limitation.

iii. The toxicity level at 60% SSL can be considered lethal to xylose pathway of the recombinant *S. cerevisiae* strains under the conditions imposed in the present study, as it did not to allow efficient xylose fermentation or xylose depletion in a batch setup (Figure 13).

iv. Fed batch fermentation (both pulse and continuous feeding) resulted in increased ethanol titre and productivity values for the fermentation of 60-65% SSL, compared to batch, as feeding allowed cells to tolerate and mitigate inhibition more efficiently (Figure 14, Figure 17).

v. Continuous feeding in a fed batch culture, coupled with the use of the novel xylose capable strain, CelluX™4, allowed the obtaining of 10-15 g/L of ethanol compared to 1-4 g/L in batch mode, 7-8 g/L in pulse feeding and 11-12 g/L from previous literature studies for non-detoxified HW-SSL. In this study, however, bioprocessing conditions were more industrially relevant as less than 1 g/L cell dry weight was pitched in culture, no pretreatment was done, bioreactor cultures were conducted non-aseptically, medium pH was never elevated above a value of 5 and an industrial nutrient source (corn steep liquor) was used. The results of this study provide a promising outlook for the utilisation of fed batch strategy and novel strains, for future studies on high TDS, non-detoxified HW-SSL fermentations.

5.2 Recommendations

Detoxification

As the current ethanol titre is still far below the industrial requirement of 40 g/L, economic compromises such as detoxification should be allowed and investigated to increase the product concentration. Xylose has been mostly unfermentable at high SSL concentration (>60%) in batch cultures due to the high inhibitor concentrations. The fermentation performance can be improved by decreasing the concentration of these inhibitors prior to fermentation by means of physical or chemical pretreatment methods. By coupling detoxification with fed batch strategy and an advanced xylose capable *S. cerevisiae* strain such as CelluX™4, the ethanol titre can be further improved.

Microbial adaptation to SSL

CelluX™4 has proven to be the most efficient strain in HW-SSL fermentations due to its high xylose affinity. The xylose capability has however proven to be sensitive to increased inhibitor concentrations, and improvement of the strain will have to entail adaptation (evolutionary engineering) to SSL, thus forming isolates with tolerance to multiple inhibitors in SSL (Kim, 2018; Taherzadeh and Karimi, 2011). Robustness can also be increased by means of rational engineering techniques, as conducted by Brandt (2019), but care should be taken to prevent the selection of robust isolates at the expense of xylose capability where growth will be prioritised over ethanol production. . Due to the vast variety of lignocellulosic fermentation feedstocks and subsequent requirements, it is best to develop a tailor-made strain with the distinct function of fermenting HW-SSL (Cunha, Romaní, et al., 2019).

Further process optimisation

The fed batch fermentation scheme can be further optimised by varying feed rates to maximise the economic return of the fermentation as a function of ethanol titre and productivity. The current dataset has proven the benefit of increasing inoculum size. Future studies can vary the cell density to obtain an efficient fermentation while still adhering to economically feasible seed production heuristics.

Supplementing hexose sugars

Although xylose is not efficiently metabolised at high inhibitor concentrations, it has been shown that glucose can still be easily fermented at 60% SSL concentration. The ethanol titre

can therefore be improved by blending the HW-SSL with other industrially relevant hexose sources such as lignocellulosic hydrolysate or sugarcane molasses.

REFERENCES

Alkasrawi, M., Rudolf, A., Lidén, G. and Zacchi, G. (2006), "Influence of strain and cultivation procedure on the performance of simultaneous saccharification and fermentation of steam pretreated spruce", *Enzyme and Microbial Technology*, Vol. 38 No. 1–2, pp. 279–286.

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G. and Gorwa-Grauslund, M.F. (2007), "Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae", *Journal of Chemical Technology and Biotechnology*, Vol. 82 No. 4, pp. 340–349.

Alriksson, B., Cavka, A. and Jönsson, L.J. (2011), "Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents", *Bioresource Technology*, Vol. 102 No. 2, pp. 1254–1263.

Ask, M., Bettiga, M., Duraiswamy, V.R. and Olsson, L. (2013), "Pulsed addition of HMF and furfural to batch-grown xylose-utilizing Saccharomyces cerevisiae results in different physiological responses in glucose and xylose consumption phase", *Biotechnology for Biofuels*, Vol. 6 No. 1, available at:https://doi.org/10.1186/1754-6834-6-181.

Ask, M., Bettiga, M., Mapelli, V. and Olsson, L. (2013), "The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing Saccharomyces cerevisiae", *Biotechnology for Biofuels*, Vol. 6 No. 1, available at:https://doi.org/10.1186/1754-6834-6-22.

Avanthi, A., Kumar, S., Sherpa, K.C. and Banerjee, R. (2017), "Bioconversion of hemicelluloses of lignocellulosic biomass to ethanol: an attempt to utilize pentose sugars", *Biofuels*, Vol. 8 No. 4, pp. 431–444.

Bajwa, P.K., Pinel, D., Martin, V.J.J., Trevors, J.T. and Lee, H. (2010), "Strain improvement of the pentose-fermenting yeast Pichia stipitis by genome shuffling", *Journal of Microbiological Methods*, Vol. 81 No. 2, pp. 179–186.

Bajwa, P.K., Shireen, T., D'Aoust, F., Pinel, D., Martin, V.J.J., Trevors, J.T. and Lee, H. (2009), "Mutants of the pentose-fermenting yeast Pichia stipitis with improved tolerance to inhibitors in hardwood spent sulfite liquor", *Biotechnology and Bioengineering*, Vol. 104 No. 5, pp. 892–900.

Bellissimi, E., Van Dijken, J.P., Pronk, J.T. and Van Maris, A.J.A. (2009), "Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based

saccharomyces cerevisiae strain", *FEMS Yeast Research*, Vol. 9 No. 3, pp. 358–364.

Bergdahl, B., Heer, D., Sauer, U., Hahn-Hägerdal, B. and Van Niel, E.W. (2012), "Dynamic metabolomics differentiates between carbon and energy starvation in recombinant saccharomyces cerevisiae fermenting xylose", *Biotechnology for Biofuels*, Vol. 5 No. 1, available at:https://doi.org/10.1186/1754-6834-5-34.

Branco, R.H.R., Serafim, L.S. and Xavier, A.M.R.B. (2019), "Second generation bioethanol production: On the use of pulp and paper industry wastes as feedstock", *Fermentation*, available at:https://doi.org/10.3390/fermentation5010004.

Brandt, B.A. (2019), *Hardening Yeast for Cellulosic Ethanol Production*, Stellenbosch University.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A. and Alfani, F. (2004), "Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF", *Process Biochemistry*, Vol. 39 No. 11, pp. 1533–1542.

Casey, E., Sedlak, M., Ho, N.W.Y. and Mosier, N.S. (2010), "Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae", *FEMS Yeast Research*, Vol. 10 No. 4, pp. 385–393.

Chandel, A.K., da Silva, S.S. and Singh, O. V. (2013), "Detoxification of Lignocellulose Hydrolysates: Biochemical and Metabolic Engineering Toward White Biotechnology", *Bioenergy Research*, Vol. 6, pp. 388–401.

Chandel, A.K., da Silva, S.S. and Singh, O. V. (2012), "Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production", *Biofuel Production-Recent Developments and Prospects*, Vol. 6 No. 1, pp. 388–401.

Chipeta, Z.A., Du Preez, J.C., Szakacs, G. and Christopher, L. (2005), "Xylanase production by fungal strains on spent sulphite liquor", *Applied Microbiology and Biotechnology*, Vol. 69 No. 1, pp. 71–78.

Chung, I.S. and Lee, Y.Y. (1985), "Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula", *Biotechnology and Bioengineering*, Vol. 27 No. 3, pp. 308–315.

Cofré, O., Ramírez, M., Gómez, J.M. and Cantero, D. (2016), "Pilot scale fed-batch fermentation in a closed loop mixed reactor for the biotransformation of crude glycerol

into ethanol and hydrogen by Escherichia coli MG1655", *Biomass and Bioenergy*, Vol. 91 No. 1, pp. 37–47.

Coote, N. and Kirsop, B.H. (1976), "FACTORS RESPONSIBLE FOR THE DECREASE IN pH DURING BEER FERMENTATIONS", *Journal of the Institute of Brewing*, Vol. 82 No. 1, pp. 149–153.

Cunha, J.T., Romaní, A., Costa, C.E., Sá-Correia, I. and Domingues, L. (2019), "Molecular and physiological basis of Saccharomyces cerevisiae tolerance to adverse lignocellulose-based process conditions", *Applied Microbiology and Biotechnology*, Vol. 103 No. 1, pp. 159–175.

Cunha, J.T., Soares, P.O., Romaní, A., Thevelein, J.M. and Domingues, L. (2019), "Xylose fermentation efficiency of industrial Saccharomyces cerevisiae yeast with separate or combined xylose reductase/xylitol dehydrogenase and xylose isomerase pathways", *Biotechnology for Biofuels*, BioMed Central, Vol. 12 No. 1, pp. 1–14.

Davey, K.R. (1994), "Modelling the combined effect of temperature and pH on the rate coefficient for bacterial growth", *International Journal of Food Microbiology*, Vol. 23 No. 1, pp. 295–303.

Delgenes, J.P., Moletta, R. and Navarro, J.M. (1996), "Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae", *Enzyme and Microbial Technology*, Vol. 19 No. 3, pp. 220–225.

Deparis, Q., Claes, A., Foulquié-Moreno, M.R. and Thevelein, J.M. (2017), "Engineering tolerance to industrially relevant stress factors in yeast cell factories", *FEMS Yeast Research*, Vol. 17 No. 4, available at:https://doi.org/10.1093/femsyr/fox036.

Giannattasio, S., Guaragnella, N., Ždralević, M. and Marra, E. (2013), "Molecular mechanisms of Saccharomyces cerevisiae stress adaptation and programmed cell death in response to acetic acid", *Frontiers in Microbiology*, Vol. 4 No. 33, available at:https://doi.org/10.3389/fmicb.2013.00033.

Hahn-Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-martins, I. and Gorwa-grauslund, M.F. (2007), "Towards industrial pentose-fermenting yeast strains", *Applied Microbiology and Biotechnology*, Vol. 74 No. 1, pp. 937–953.

Hahn-Hägerdal, B. and Palmqvist, E. (2000), "Fermentation of lignocellulosic hydrolysates.

II: Inhibitors and mechanisms of inhibition", *Bioresource Technology*, Vol. 74 No. 1, pp. 25–33.

Hamelinck, C.N., Van Hooijdonk, G. and Faaij, A.P.C. (2005), "Ethanol from lignocellulosic biomass: Techno-economic performance in short-, middle- and long-term", *Biomass and Bioenergy*, Vol. 28 No. 4, pp. 384–410.

Han, X., Liu, G., Song, W., Qin, Y. and Qu, Y. (2017), "Continuous feeding of spent ammonium sulphite liquor improves the production and saccharification performance of cellulase by Penicillium oxalicum", *Bioresource Technology*, Vol. 245, pp. 984–992.

Harner, N.K., Bajwa, P.K., Formusa, P.A., Austin, G.D., Habash, M.B., Trevors, J.T., Chan, C.K., et al. (2015), "Determinants of tolerance to inhibitors in hardwood spent sulfite liquor in genome shuffled Pachysolen tannophilus strains", *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, Vol. 108 No. 1, pp. 811–834.

Harner, N.K., Bajwa, P.K., Habash, M.B., Trevors, J.T., Austin, G.D. and Lee, H. (2014), "Mutants of the pentose-fermenting yeast Pachysolen tannophilus tolerant to hardwood spent sulfite liquor and acetic acid", *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, Vol. 105 No. 1, pp. 29–43.

Heer, D. and Sauer, U. (2008), "Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain", *Microbial Biotechnology*, Vol. 6 No. 1, pp. 497–506.

Helle, S.S., Lin, T. and Duff, S.J.B. (2008), "Optimization of spent sulfite liquor fermentation", *Enzyme and Microbial Technology*, Vol. 42 No. 3, pp. 259–264.

Helle, S.S., Murray, A., Lam, J., Cameron, D.R. and Duff, S.J.B. (2004), "Xylose fermentation by genetically modified Saccharomyces cerevisiae 259ST in spent sulfite liquor", *Bioresource Technology*, Vol. 92 No. 2, pp. 163–171.

Henriques, T.M., Pereira, S.R., Serafim, L.S. and Xavier, A.M.R.B. (2018), "Two-stage aeration fermentation strategy to improve bioethanol production by Scheffersomyces stipitis", *Fermentation*, Vol. 4 No. 4, available at:https://doi.org/10.3390/fermentation4040097.

Holderby, J.M. and Moggio, W.A. (1960), "Utilization of Spent Sulfite Liquor", *Journal (Water Pollution Control Federation)*, Vol. 32 No. 2, pp. 171–181.

Huang, H., Guo, X., Li, D., Liu, M., Wu, J. and Ren, H. (2011), "Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids", *Bioresource Technology*, Vol. 102 No. 16, pp. 7486–7493.

Johansson, E., Brandberg, T. and Larsson, C. (2011), "Influence of cultivation procedure for Saccharomyces cerevisiae used as pitching agent in industrial spent sulphite liquor fermentations", *Journal of Industrial Microbiology and Biotechnology*, Vol. 38 No. 11, pp. 1787–1792.

Johansson, E., Xiros, C. and Larsson, C. (2014), "Fermentation performance and physiology of two strains of Saccharomyces cerevisiae during growth in high gravity spruce hydrolysate and spent sulphite liquor", *BMC Biotechnology*, Vol. 14 No. 1, pp. 1–17.

Jonsson, L., Alriksson, B. and Nilvebrant, N. (2013), "Bioconversion of lignocellulose: inhibitors and detoxification", *Biotechnology for Biofuels*, Vol. 6 No. 1, pp. 1–10.

Jönsson, L.J. and Martín, C. (2016), "Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects", *Bioresource Technology*, Vol. 199 No. 1, pp. 103–112.

Jönsson, L.J., Palmqvist, E., Nilvebrant, N.O. and Hahn-Hägerdal, B. (1998), "Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor", *Applied Microbiology and Biotechnology*, Vol. 49 No. 1, pp. 691–697.

Jørgensen, H. (2009), "Effect of nutrients on fermentation of pretreated wheat straw at very high dry matter content by saccharomyces cerevisiae", *Applied Biochemistry and Biotechnology*, Vol. 153 No. 1, pp. 44–57.

Karapatsia, A., Penloglou, G., Chatzidoukas, C. and Kiparissides, C. (2016), "Fed-batch Saccharomyces cerevisiae fermentation of hydrolysate sugars: A dynamic model-based approach for high yield ethanol production", *Biomass and Bioenergy*, Vol. 90 No. 1, pp. 32–41.

Kim, D. (2018), "Physico-chemical conversion of lignocellulose: Inhibitor effects and detoxification strategies: A mini review", *Molecules*, Vol. 23 No. 2, p. 309.

Klinke, H.B., Olsson, L., Thomsen, A.B. and Ahring, B.K. (2003), "Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: Wet oxidation and fermentation by yeast", *Biotechnology and Bioengineering*, Vol. 81 No. 6, pp. 738–747.

Ko, J.K., Enkh-Amgalan, T., Gong, G., Um, Y. and Lee, S.-M. (2020), "Improved bioconversion of lignocellulosic biomass by Saccharomyces cerevisiae engineered for tolerance to acetic acid", *GCB Bioenergy*, Vol. 12 No. 1, pp. 90–100.

Ko, J.K., Um, Y. and Lee, S.M. (2016), "Effect of manganese ions on ethanol fermentation by xylose isomerase expressing Saccharomyces cerevisiae under acetic acid stress", *Bioresource Technology*, Vol. 222 No. 1, pp. 422–430.

Ko, J.K., Um, Y., Woo, H.M., Kim, K.H. and Lee, S.M. (2016), "Ethanol production from lignocellulosic hydrolysates using engineered Saccharomyces cerevisiae harboring xylose isomerase-based pathway", *Bioresource Technology*, Vol. 209 No. 1, pp. 290–296.

Kuyper, M., Toirkens, M.J., Diderich, J.A., Winkler, A.A., Van Dijken, J.P. and Pronk, J.T. (2005), "Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting Saccharomyces cerevisiae strain", *FEMS Yeast Research*, Vol. 5 No. 10, pp. 925–934.

Lane, S., Xu, H., Oh, E.J., Kim, H., Lesmana, A., Jeong, D., Zhang, G., et al. (2018), "Glucose repression can be alleviated by reducing glucose phosphorylation rate in Saccharomyces cerevisiae", *Scientific Reports*, Vol. 8 No. 1, available at:https://doi.org/10.1038/s41598-018-20804-4.

Lawford, H.G. and Rousseau, J.D. (1993), "Production of ethanol from pulp mill hardwood and softwood spent sulfite liquors by genetically engineered E. coli", *Applied Biochemistry and Biotechnology*, Vol. 39 No. 1, pp. 667–685.

Li, Y.C., Gou, Z.X., Zhang, Y., Xia, Z.Y., Tang, Y.Q. and Kida, K. (2017), "Inhibitor tolerance of a recombinant flocculating industrial Saccharomyces cerevisiae strain during glucose and xylose co-fermentation", *Brazilian Journal of Microbiology*, Vol. 48 No. 4, pp. 791–800.

Liu, Y., Yang, S. and Jia, X. (2020), "Construction of a 'nutrition supply–detoxification' coculture consortium for medium-chain-length polyhydroxyalkanoate production with a glucose–xylose mixture", *Journal of Industrial Microbiology and Biotechnology*, Vol. 47 No. 1, pp. 343–354.

Liu, Z.L., Huang, X., Zhou, Q. and Xu, J. (2019), "Protein expression analysis revealed a fine-tuned mechanism of in situ detoxification pathway for the tolerant industrial yeast Saccharomyces cerevisiae", *Applied Microbiology and Biotechnology*, Vol. 103 No. 1, pp. 5781–5796.

Llano, T., Alexandri, M., Koutinas, A., Gardeli, C., Papapostolou, H., Coz, A., Quijorna, N., et al. (2015), "Liquid–Liquid Extraction of Phenolic Compounds from Spent Sulphite Liquor", *Waste and Biomass Valorization*, Springer Netherlands, Vol. 6 No. 6, pp. 1149–1159.

Llano, T., Quijorna, N. and Coz, A. (2017), "Detoxification of a lignocellulosic waste from a pulp mill to enhance its fermentation prospects", *Energies*, Vol. 10 No. 3, available at:https://doi.org/10.3390/en10030348.

Marques, A.P., Evtuguin, D. V., Magina, S., Amado, F.M.L. and Prates, A. (2009), "Chemical composition of Spent liquors from acidic magnesium-based Sulphite pulping of Eucalyptus globulus", *Journal of Wood Chemistry and Technology*, Vol. 29 No. 4, pp. 322–336.

Martinez, A., Rodriguez, M.E., Wells, M.L., York, S.W., Preston, J.F. and Ingram, L.O. (2001), "Detoxification of dilute acid hydrolysates of lignocellulose with lime", *Biotechnology Progress*, Vol. 17 No. 2, pp. 287–293.

Matsushika, A., Goshima, T. and Hoshino, T. (2014), "Transcription analysis of recombinant industrial and laboratory Saccharomyces cerevisiae strains reveals the molecular basis for fermentation of glucose and xylose", *Microbial Cell Factories*, Vol. 13 No. 16, available at:https://doi.org/10.1186/1475-2859-13-16.

Matsushika, A., Nagashima, A., Goshima, T. and Hoshino, T. (2013), "Fermentation of Xylose Causes Inefficient Metabolic State Due to Carbon/Energy Starvation and Reduced Glycolytic Flux in Recombinant Industrial Saccharomyces cerevisiae", *PLoS ONE*, Vol. 8 No. 7, available at:https://doi.org/10.1371/journal.pone.0069005.

Meinander, N.Q. and Hahn-Hägerdal, B. (1997), "Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing Saccharomyces cerevisiae: A comparison of different sugars and ethanol as cosubstrates", *Applied and Environmental Microbiology*, Vol. 63 No. 5, pp. 1959–1964.

Modig, T., Almeida, J.R.M., Gorwa-Grauslund, M.F. and Lidén, G. (2008), "Variability of the response of Saccharomyces cerevisiae strains to lignocellulose hydrolysate", *Biotechnology and Bioengineering*, Vol. 100 No. 3, pp. 423–429.

Mokomele, T. (2019), *Integrated Bioenergy and Animal Feed Production from AFEX^{TM} and Steam Exploded Sugarcane*, Stellenbosch University.

Moysés, D.N., Reis, V.C.B., de Almeida, J.R.M., de Moraes, L.M.P. and Torres, F.A.G. (2016), "Xylose fermentation by saccharomyces cerevisiae: Challenges and prospects", *International Journal of Molecular Sciences*, Vol. 17 No. 3, available at:https://doi.org/10.3390/ijms17030207.

Nigam, J.N. (2001), "Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis", *Journal of Industrial Microbiology and Biotechnology*, Vol. 26 No. 1, pp. 145–150.

Nilsson, A., Taherzadeh, M.J. and Lidén, G. (2001), "Use of dynamic step response for control of fed-batch conversion of lignocellulosic hydrolyzates to ethanol", *Journal of Biotechnology*, Vol. 89 No. 1, pp. 41–53.

Nilvebrant, N.-O., Persson, P., Reimann, A., de Sousa, F., Gorton, L. and Jönsson, L.J. (2003), "Limits for Alkaline Detoxification of Dilute-Acid Lignocellulose Hydrolysates", *Biotechnology for Fuels and Chemicals*, Vol. 107 No. 3, pp. 615–628.

Novy, V., Krahulec, S., Longus, K., Klimacek, M. and Nidetzky, B. (2013), "Co-fermentation of hexose and pentose sugars in a spent sulfite liquor matrix with genetically modified Saccharomyces cerevisiae", *Bioresource Technology*, Vol. 107 No. 1, pp. 439–448.

Ogden, J.M., Hochgreb, S. and Hylton, M. (1990), "International sugar journal.", *International Sugar Journal*, Vol. 92 No. 1099, pp. 131–140.

Palmqvist, E., Grage, H., Meinander, N.Q. and Hahn-Hägerdal, B. (1999), "Main and interaction effects of acetic acid, furfural, and p- hydroxybenzoic acid on growth and ethanol productivity of yeasts", *Biotechnology and Bioengineering*, Vol. 61 No. 1, pp. 43–55.

Pampulha, M.E. and Loureiro-Dias, M.C. (1989), "Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast", *Applied Microbiology and Biotechnology*, Vol. 31 No. 1, pp. 547–550.

Parawira, W. and Tekere, M. (2011), "Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: Review", *Critical Reviews in Biotechnology*, Vol. 31 No. 1, pp. 30–31.

Pereira, S.R., Ivanuša, Š., Evtuguin, D. V., Serafim, L.S. and Xavier, A.M.R.B. (2012), "Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol", *Bioresource Technology*, Vol. 103 No. 1, pp. 131–135.

Pereira, S.R., Portugal-nunes, D.J., Evtuguin, D. V, Serafim, L.S. and Xavier, A.M.R.B. (2013), "Advances in ethanol production from hardwood spent sulphite liquors", *Process Biochemistry*, Elsevier Ltd, Vol. 48 No. 2, pp. 272–282.

Pereira, S.R., Sànchez I Nogué, V., Frazão, C.J.R., Serafim, L.S., Gorwa-Grauslund, M.F. and Xavier, A.M.R.B. (2015), "Adaptation of Scheffersomyces stipitis to hardwood spent sulfite liquor by evolutionary engineering", *Biotechnology for Biofuels*, Vol. 8 No. 1, available at:https://doi.org/10.1186/s13068-015-0234-y.

Petersen, A.M., Haigh, K. and Görgens, J.F. (2014), "Techno-economics of integrating bioethanol production from spent sulfite liquor for reduction of greenhouse gas emissions from sulfite pulping mills", *Biotechnology for Biofuels*, Vol. 7 No. 1, pp. 1–14.

Pienkos, P.T. and Zhang, M. (2009), "Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates", *Cellulose*, Vol. 16 No. 1, pp. 743–762.

Pinel, D., D'Aoust, F., del Cardayre, S.B., Bajwa, P.K., Lee, H. and Martin, V.J.J. (2011), "Saccharomyces cerevisiae genome shuffling through recursive population mating leads to improved tolerance to spent sulfite liquor", *Applied and Environmental Microbiology*, Vol. 77 No. 14, pp. 4736–4743.

Rivard, C.J., Engel, R.E., Hayward, T.K., Nagle, N.J., Hatzis, C. and Philippidis, G.P. (1996), "Measurement of the inhibitory potential and detoxification of biomass pretreatment hydrolysate for ethanol production", *Applied Biochemistry and Biotechnology*, Vol. 57 No. 1, pp. 89–95.

Rueda, C., Calvo, P.A., Moncali??n, G., Ruiz, G. and Coz, A. (2015), "Biorefinery options to valorize the spent liquor from sulfite pulping", *Journal of Chemical Technology and Biotechnology*, Vol. 90 No. 12, pp. 2218–2226.

Schimz, K.L. (1980), "The effect of sulfite on the yeast Saccharomyces cerevisiae", *Archives of Microbiology*, Vol. 125 No. 1, pp. 59–95.

Shuler, M.L., Kargi, F. and DeLisa, M. (2017), *Bioprocess Bngineering: Basic Concepts*.

Soares, L.B., Nakanishi, S.C., Rocha, G.J.M., Biazi, L.E., Ienczak, J.L., Nascimento, V.M. and Costa, A.C. (2017), " Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by Spathaspora passalidarum and Scheffersomyces stipitis ", *Biotechnology and Bioengineering*, Vol. 114 No. 10, pp. 2211–2221.

Souto-Maior, A.M., Runquist, D. and Hahn-Hägerdal, B. (2009), "Crabtree-negative

characteristics of recombinant xylose-utilizing Saccharomyces cerevisiae", *Journal of Biotechnology*, Vol. 143 No. 2, pp. 119–123.

Subtil, T. and Boles, E. (2012), "Competition between pentoses and glucose during uptake and catabolism in recombinant Saccharomyces cerevisiae", *Biotechnology for Biofuels*, Vol. 5 No. 1, available at:https://doi.org/10.1186/1754-6834-5-14.

Taherzadeh, M.J. and Karimi, K. (2011), "Fermentation inhibitors in ethanol processes and different strategies to reduce their effects", *Biofuels*, pp. 287–311.

Taherzadeh, M.J., Niklasson, C. and Lidén, G. (1999), "Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation", *Bioresource Technology*, Vol. 69 No. 1, pp. 59–66.

Taherzadeh, M.J., Niklasson, C. and Lidén, G. (2000), "On-line control of fed-batch fermentation of dilute-acid hydrolyzates", *Biotechnology and Bioengineering*, Vol. 69 No. 3, pp. 330–338.

Wang, H., Li, Q., Kuang, X., Xiao, D., Han, X., Hu, X., Li, X., et al. (2018), "Functions of aldehyde reductases from Saccharomyces cerevisiae in detoxification of aldehyde inhibitors and their biotechnological applications", *Applied Microbiology and Biotechnology*, Vol. 102 No. 1, pp. 10439–10456.

Wang, S., Sun, X. and Yuan, Q. (2018), "Strategies for enhancing microbial tolerance to inhibitors for biofuel production: A review", *Bioresource Technology*, Vol. 258 No. 1, pp. 302–309.

Wang, X., Jin, M., Balan, V., Jones, A.D., Li, X., Li, B.Z., Dale, B.E., et al. (2014), "Comparative metabolic profiling revealed limitations in xylose-fermenting yeast during co-fermentation of glucose and xylose in the presence of inhibitors", *Biotechnology and Bioengineering*, Vol. 111 No. 1, pp. 152–164.

Wei, S., Liu, Y., Wu, M., Ma, T., Bai, X., Hou, J., Shen, Y., et al. (2018), "Disruption of the transcription factors Thi2p and Nrm1p alleviates the post-glucose effect on xylose utilization in Saccharomyces cerevisiae", *Biotechnology for Biofuels*, Vol. 11 No. 1, available at:https://doi.org/10.1186/s13068-018-1112-1.

Wingren, A., Galbe, M. and Zacchi, G. (2003), "Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks", *Biotechnology Progress*, Vol. 19 No. 4, pp. 1109–1117.

Xavier, A.M.R.B., Correia, M.F., Pereira, S.R. and Evtuguin, D. V. (2010), "Second-generation bioethanol from eucalypt sulphite spent liquor", *Bioresource Technology*, Vol. 101 No. 8, pp. 2755–2761.

Zhang, G.C., Kong, I.I., Wei, N., Peng, D., Turner, T.L., Sung, B.H., Sohn, J.H., et al. (2016), "Optimization of an acetate reduction pathway for producing cellulosic ethanol by engineered yeast", *Biotechnology and Bioengineering*, Vol. 113 No. 12, pp. 2587–2596.

Zhang, J., Zhang, W.X., You, L., Yin, L.G., Du, Y.H. and Yang, J. (2014), "Modified method combining in situ detoxification with simultaneous saccharification and cofermentation (SSCF) as a single step for converting exploded rice straw into ethanol", *Journal of Agricultural and Food Chemistry*, Vol. 60 No. 30, pp. 7486–7495.

Zhu, J.Q., Li, X., Qin, L., Li, W.C., Li, H.Z., Li, B.Z. and Yuan, Y.J. (2016), "In situ detoxification of dry dilute acid pretreated corn stover by co-culture of xylose-utilizing and inhibitor-tolerant Saccharomyces cerevisiae increases ethanol production", *Bioresource Technology*, Vol. 218 No. 1, pp. 380–387.

APPENDIX

Fermentation of low TDS SSL (Mg-4)

The fermentation of high TDS SSL streams such as Mg-1A is often unfeasible as is, due to the high solids loading and relating inhibition issues (Petersen et al., 2014; Taherzadeh and Karimi, 2011). In this study these high solid inhibition effect will be mitigated by ultimately utilising the high TDS SSL in a fed batch fermentation. From a practical viewpoint it is however necessary to initiate this fermentation strategy with a successful batch phase, which can only be obtained in sufficiently low inhibitor concentrations. All SSL streams contain more than 14 g/L acetic acid, which is theoretically too high to allow efficient xylose fermentation.

To conceptually test the feasibility of fermenting an industrial stream as is, the performance of the recombinant strain CelluX™4 was assessed in the Mg-4 SSL stream in batch configuration. Although recombinant *S. cerevisiae* strains with the XI-pathway are more capable in oxygen limited conditions than those with XR/XDH pathway (Cunha, Soares, et al., 2019; Zhang et al., 2016), the consumption of acetic acid and furfural could lead to co-factor imbalances (Ask, Bettiga, Duraiswamy, et al., 2013; Ask, Bettiga, Mapelli, et al., 2013; Zhang et al., 2016). To determine the effect of oxygen on the fermentation, rubber stoppers and cotton plugs were used to create microaerophilic and semi-aerobic conditions, respectively. Figure 19 and Table 15 display the fermentation profile and kinetic parameters obtained in the fermentation.

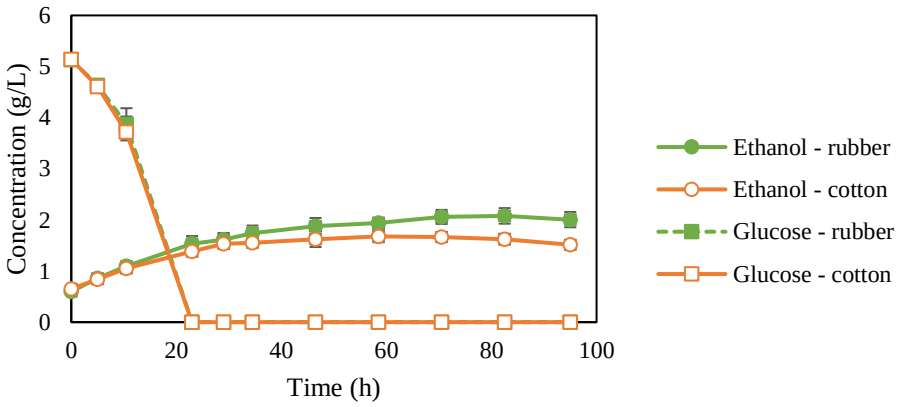

Figure 19: Fermentation profiles of Mg-4 SSL in batch culture under different oxygen conditions facilitated by rubber and cotton stoppers respectively

Table 15: Fermentation parameters of Mg-4 SSL in batch culture under different oxygen conditions

Conditions	**Glucose$_i$ (g/L)**	**Xylose$_i$ (g/L)**	**Xylose$_f$ (g/L)**	**Ethanol$_{max}$ (g/L)**	**Productivity (g/Lh)**	**$Y_{P/S}$ (g/g)**
Micro-aerophilic	5.1 ± 0.1	31.7 ± 0.4	28.8 ± 0.5	1.46 ± 0.15	0.021 ± 0.002	0.16 ± 0.01
Semi-aerobic	5.1 ± 0.1	30.3 ± 0.3	30.3 ± 0.4	1.02 ± 0.13	0.018 ± 0.002	0.18 ± 0.01

It is clear from Table 15 that extremely low amounts of ethanol, in the range of 1-1.5 g/L, was produced from the low TDS SSL stream in both microaerophilic and semi-aerobic conditions. Not only did the fermentations obtain an ethanol yield of less than 40%, but negligible amounts of xylose were consumed. Ethanol was therefore mainly produced by the glucose section of the medium. Fermentations under microaerophilic conditions produced marginally higher amounts of ethanol than that of semi-aerobic conditions. Figure 20 displays the profiles of furfural and acetic acid in the fermentations of Mg-4 SSL.

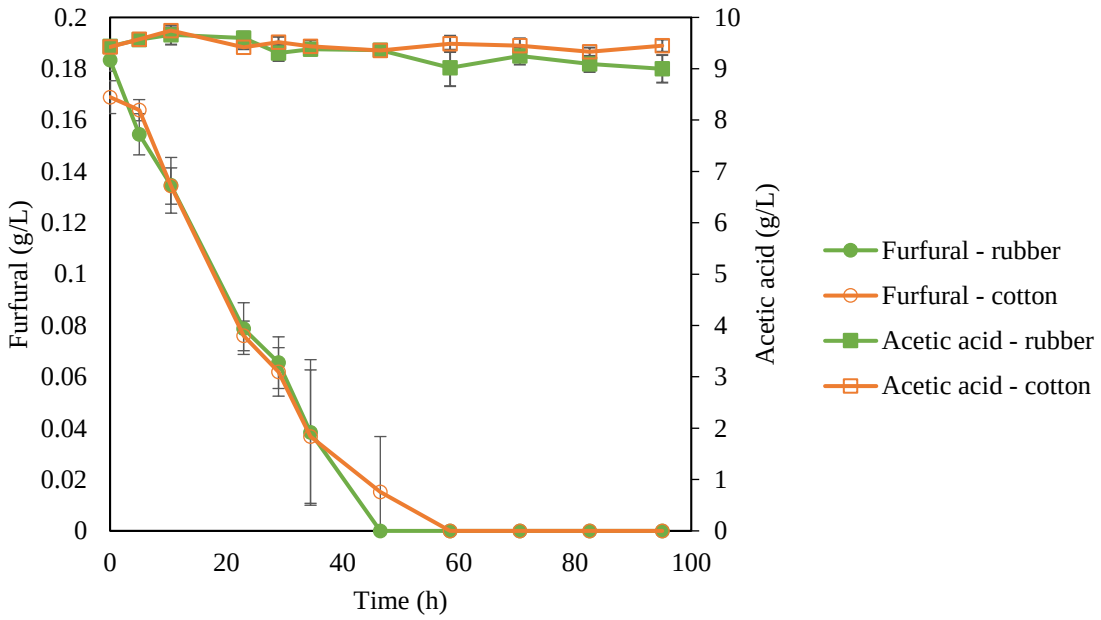

Figure 20: Inhibitor degradation during the fermentation of Mg-4 SSL facilitated by rubber and cotton stoppers respectively

Figure 20 indicates that furfural could be consumed at similar rates under both microaerophilic and semi-aerobic conditions. Acetic acid on the other hand remained at 9-10 g/L, regardless of oxygen conditions.

It is clear from both ethanol and inhibitor data that the use of cotton wool stoppers holds no benefit over that of rubber stoppers. Thin SSL is however too inhibitor rich to allow efficient batch fermentation. A contributing factor to this poor performance is the high acetic acid concentration in low TDS SSL (>10 g/L), since it is known to impede xylose metabolism greatly as shown in Figure 2 in the literature section. Shake flask batch fermentations employed in the strain screening process should therefore be conducted with Mg 1A SSL which has been diluted down with R/O water and rubber stoppers.

Raw data

Table 16: CelluX^{TM}4 culture in synthetic media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.00	0.00	0.00	4.36	4.19	4.25	26.58	25.79	26.20
2.50	0.00	0.00	0.00	4.09	4.04	4.05	26.53	26.42	26.57
4.50	0.67	0.73	0.85	3.02	3.14	2.86	26.27	26.53	25.35
8.00	1.99	2.04	2.01	1.10	0.93	0.97	24.26	24.66	24.43
12.00	3.32	3.25	3.28	0.00	0.00	0.00	22.21	22.56	22.10
23.50	5.91	5.81	5.50	0.00	0.00	0.00	15.60	15.80	15.34
29.00	7.18	7.48	5.50	0.00	0.00	0.00	12.64	12.59	9.47
35.00	9.00	9.10	8.72	0.00	0.00	0.00	9.27	8.65	8.41
47.00	11.88	11.70	11.39	0.00	0.00	0.00	1.97	1.63	1.43
53.00	12.21	12.08	11.67	0.00	0.00	0.00	0.88	0.73	0.62
61.00	9.66	12.38	9.28	0.00	0.00	0.00	0.00	0.00	0.00
72.00	12.43	12.09	11.94	0.00	0.00	0.00	0.00	0.00	0.00
83.00	12.34	12.15	11.98	0.00	0.00	0.00	0.00	0.00	0.00
95.00	12.19	11.96	11.53	0.00	0.00	0.00	0.00	0.00	0.00
107.00	12.91	12.35	12.11	0.00	0.00	0.00	0.00	0.00	0.00

Table 17: TFA7 culture in synthetic media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.48	0.53	0.56	4.29	4.29	4.26	26.27	25.86	25.42
2.50	0.87	0.89	0.82	3.47	3.46	3.40	25.29	25.64	25.54
4.50	1.61	1.61	1.73	1.91	1.77	1.77	24.68	24.51	24.61
8.00	2.61	2.78	2.61	0.00	0.00	0.00	22.93	22.87	23.04
12.00	3.22	3.32	3.26	0.00	0.00	0.00	22.15	22.08	21.53
23.50	3.63	3.73	3.79	0.00	0.00	0.00	19.61	20.05	19.54
29.00	3.98	3.89	4.09	0.00	0.00	0.00	19.20	18.71	18.60
35.00	4.65	4.32	3.87	0.00	0.00	0.00	18.95	18.14	17.83
47.00	4.70	4.69	4.53	0.00	0.00	0.00	17.23	17.59	14.89
53.00	4.84	4.91	4.65	0.00	0.00	0.00	17.05	17.06	14.24
61.00	5.06	5.22	4.89	0.00	0.00	0.00	15.94	16.01	12.39
72.00	5.32	5.35	5.42	0.00	0.00	0.00	15.18	15.11	10.97
83.00	5.78	5.75	5.45	0.00	0.00	0.00	14.43	14.34	9.84
95.00	5.95	5.70	6.00	0.00	0.00	0.00	13.52	13.30	8.80
107.00	6.29	6.11	3.27	0.00	0.00	0.00	13.29	12.52	4.96

Table 18: CelluX^{TM}4 culture in 20% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.26	0.56	0.50	2.23	2.32	2.38	15.09	15.67	16.11	2.07	2.19	2.15
19.00	1.51	1.81	2.71	0.00	0.00	0.00	13.08	10.46	10.47	1.47	0.80	1.48
44.00	2.65	3.14	4.29	0.00	0.00	0.00	9.27	5.99	5.50	1.08	0.29	1.30
68.00	3.92	4.04	5.17	0.00	0.00	0.00	6.19	2.81	3.12	0.96	0.00	1.25
92.00	4.14	3.75	5.37	0.00	0.00	0.00	3.42	0.00	0.00	0.80	0.00	1.10
116.00	4.10	3.20	5.04	0.00	0.00	0.00	2.39	0.00	0.00	0.52	0.00	0.77
142.00	4.13	2.77	5.19	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.70
164.00	3.96	2.32	5.09	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00	0.48

Table 19: TP1 culture in 20% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.00	0.00	0.00	2.14	2.06	2.19	14.94	13.98	15.54	2.04	1.85	2.09
19.00	1.27	2.18	1.87	0.00	0.00	0.00	11.38	10.89	11.37	0.00	1.01	1.20
44.00	0.61	2.38	2.26	0.00	0.00	0.00	8.79	7.07	10.06	0.00	0.00	1.02
68.00	0.28	2.28	2.60	0.00	0.00	0.00	7.21	5.82	8.64	0.00	0.00	0.75
92.00	0.00	2.34	2.83	0.00	0.00	0.00	5.25	5.36	7.66	0.00	0.00	0.45
116.00	0.00	2.52	3.07	0.00	0.00	0.00	3.93	3.97	6.48	0.00	0.00	0.30
142.00	0.00	1.85	3.07	0.00	0.00	0.00	3.22	2.70	5.27	0.00	0.00	0.00
164.00	0.00	1.42	3.16	0.00	0.00	0.00	2.58	0.00	4.37	0.00	0.00	0.00

Table 20: TFA7 culture in 20% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.70	0.77	0.72	2.19	2.19	2.15	14.94	15.50	15.03	2.06	2.06	2.06
19.00	2.67	2.94	2.64	0.00	0.00	0.00	10.96	10.98	10.42	1.23	1.22	0.60
44.00	3.15	2.46	3.44	0.00	0.00	0.00	8.72	7.58	6.68	1.03	0.00	0.00
68.00	3.61	2.75	3.82	0.00	0.00	0.00	7.69	3.92	3.59	0.89	0.00	0.00
92.00	3.90	2.76	3.95	0.00	0.00	0.00	6.55	3.10	2.74	0.53	0.00	0.00
116.00	4.39	2.94	3.98	0.00	0.00	0.00	4.55	2.45	0.00	0.42	0.00	0.00
142.00	4.62	2.69	3.90	0.00	0.00	0.00	3.32	0.00	0.00	0.20	0.00	0.00
164.00	4.64	2.40	3.75	0.00	0.00	0.00	2.47	0.00	0.00	0.00	0.00	0.00

Table 21: CelluX^{TM}4 culture in 40% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.79	0.82	0.88	4.80	4.77	4.97	26.99	26.53	27.81	4.13	4.11	4.26
7.50	2.72	3.27	3.09	0.00	0.00	0.00	25.36	27.14	25.88	4.10	4.53	4.27
11.50	3.52	3.96	3.82	0.00	0.00	0.00	22.70	23.99	23.15	3.94	4.15	4.03
24.50	4.64	4.91	5.10	0.00	0.00	0.00	20.07	20.46	20.27	3.76	3.95	3.90
30.50	5.05	5.22	5.41	0.00	0.00	0.00	20.03	20.50	19.58	3.91	3.97	3.85
36.00	4.79	5.22	5.67	0.00	0.00	0.00	19.51	20.38	18.98	3.56	3.90	3.84
48.00	5.68	5.87	6.05	0.00	0.00	0.00	16.65	18.61	18.19	3.36	3.92	3.85
60.00	6.08	5.99	6.31	0.00	0.00	0.00	16.14	18.01	16.45	3.30	3.91	3.80
72.00	6.36	6.23	6.42	0.00	0.00	0.00	13.96	16.21	14.76	3.17	3.71	3.51
84.00	6.47	6.65	7.33	0.00	0.00	0.00	13.21	16.04	15.21	3.17	3.93	3.91
96.00	6.95	6.83	7.13	0.00	0.00	0.00	12.22	14.71	13.54	3.25	3.84	3.68
108.00	7.04	7.14	7.39	0.00	0.00	0.00	11.53	14.03	12.05	3.13	3.88	3.68
121.00	6.98	7.42	7.59	0.00	0.00	0.00	9.69	13.99	11.51	2.64	3.91	3.68
132.00	7.66	7.53	7.86	0.00	0.00	0.00	8.04	12.24	10.76	2.42	3.84	3.70
145.00	8.16	7.46	8.05	0.00	0.00	0.00	5.77	10.89	9.74	2.37	3.75	3.64
169.50	8.62	7.82	8.38	0.00	0.00	0.00	2.90	9.19	8.18	2.11	3.45	3.44
193.00	8.98	8.02	8.35	0.00	0.00	0.00	0.00	8.21	6.99	2.22	3.53	3.46
220.00	9.29	8.16	8.02	0.00	0.00	0.00	0.00	7.49	8.21	2.19	3.54	3.53

Table 22: TP1 culture in 40% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.63	0.68	0.65	4.96	4.91	5.12	27.84	27.83	28.73	4.26	4.21	4.39
7.50	2.34	2.31	2.28	0.00	0.00	0.00	26.63	25.90	26.69	4.03	3.94	4.04
11.50	2.76	2.77	2.64	0.00	0.00	0.00	25.52	25.42	25.30	3.96	3.93	3.90
24.50	3.41	3.39	3.34	0.00	0.00	0.00	23.86	22.91	23.54	3.88	3.75	3.80
30.50	3.68	3.59	3.60	0.00	0.00	0.00	23.33	22.49	22.93	3.81	3.67	3.77
36.00	3.78	3.69	3.57	0.00	0.00	0.00	22.68	21.48	23.14	3.75	3.50	3.60
48.00	4.00	3.85	3.98	0.00	0.00	0.00	23.55	21.59	23.08	3.72	3.33	3.62
60.00	3.94	4.01	4.14	0.00	0.00	0.00	21.73	20.33	22.29	3.35	3.29	3.83
72.00	4.24	4.11	4.10	0.00	0.00	0.00	20.96	19.17	20.36	3.38	2.87	3.33
84.00	4.43	4.22	4.43	0.00	0.00	0.00	21.03	19.22	21.53	3.38	2.93	3.37
96.00	4.13	4.52	4.42	0.00	0.00	0.00	18.65	18.95	19.33	2.99	2.77	3.13
108.00	4.76	4.58	4.64	0.00	0.00	0.00	19.35	17.90	19.09	3.19	2.62	3.04
121.00	4.80	4.78	4.80	0.00	0.00	0.00	19.15	16.37	18.02	3.02	2.61	3.07
132.00	4.90	4.89	4.80	0.00	0.00	0.00	18.57	15.76	18.19	3.02	2.46	2.97
145.00	5.15	5.20	5.15	0.00	0.00	0.00	17.66	15.10	16.94	2.98	2.30	2.96
169.50	5.17	5.38	5.19	0.00	0.00	0.00	16.04	13.83	16.75	2.61	1.94	2.55
193.00	5.37	5.48	5.36	0.00	0.00	0.00	15.41	12.98	15.53	2.49	1.82	2.43
220.00	5.57	5.63	5.50	0.00	0.00	0.00	13.97	12.03	14.35	2.23	1.64	2.39

Table 23: TFA7 culture in 40% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)			Acetic acid (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.92	0.93	0.91	5.07	4.99	4.94	28.70	27.77	27.72	4.35	4.34	4.26
7.50	2.60	2.78	2.65	0.00	0.00	0.00	24.71	25.29	25.38	3.87	3.98	3.94
11.50	3.28	2.22	3.46	0.00	0.00	0.00	23.60	24.61	25.03	3.80	3.51	3.97
24.50	4.24	3.20	3.98	0.00	0.00	0.00	22.04	22.32	21.47	3.75	3.02	3.60
30.50	4.45	3.40	4.42	0.00	0.00	0.00	21.96	22.04	23.22	3.70	2.93	3.72
36.00	4.60	3.50	4.29	0.00	0.00	0.00	21.46	22.90	20.78	3.65	2.79	3.47
48.00	4.83	3.68	4.70	0.00	0.00	0.00	20.91	21.94	20.87	3.54	2.66	3.49
60.00	5.05	3.97	4.78	0.00	0.00	0.00	21.33	21.53	20.93	3.45	2.53	3.35
72.00	5.29	3.95	4.97	0.00	0.00	0.00	19.40	19.11	19.68	3.24	2.17	3.16
84.00	5.49	4.18	5.03	0.00	0.00	0.00	18.75	19.33	18.50	3.04	2.12	2.93
96.00	5.66	4.42	5.24	0.00	0.00	0.00	17.86	19.05	18.96	2.98	2.10	2.94
108.00	5.70	4.58	5.46	0.00	0.00	0.00	16.96	17.73	17.17	2.80	1.84	2.79
121.00	5.93	4.68	5.49	0.00	0.00	0.00	16.75	16.89	17.62	2.81	1.84	2.78
132.00	6.14	4.91	5.64	0.00	0.00	0.00	16.02	16.33	15.99	2.72	1.61	2.63
145.00	6.18	4.94	5.87	0.00	0.00	0.00	15.32	14.96	15.85	2.31	1.31	2.11
169.50	6.26	5.14	6.30	0.00	0.00	0.00	13.93	15.39	12.62	2.13	1.19	1.75
193.00	6.52	5.28	6.65	0.00	0.00	0.00	12.86	13.41	11.10	2.04	1.10	1.50
220.00	6.74	5.51	7.11	0.00	0.00	0.00	12.33	12.28	10.28	1.93	0.86	1.53

Table 24: CelluX^{TM}4 culture in 60% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.73	0.76	0.77	6.99	7.06	7.22	40.91	40.91	41.57
7.50	1.36	1.41	1.43	6.29	5.50	5.53	41.40	41.47	40.39
11.50	1.69	1.85	1.81	5.59	0.00	0.00	39.74	41.10	41.73
24.50	2.64	2.64	2.64	0.00	0.00	0.00	39.02	39.33	39.89
30.50	3.22	3.43	3.15	0.00	0.00	0.00	38.42	39.58	37.93
36.00	3.22	3.39	3.27	0.00	0.00	0.00	38.10	38.44	37.16
48.00	3.66	3.63	3.66	0.00	0.00	0.00	37.57	37.66	37.11
60.00	4.01	3.64	3.96	0.00	0.00	0.00	35.48	33.02	33.81
72.00	3.98	4.12	3.92	0.00	0.00	0.00	34.12	34.75	33.69
96.00	4.10	4.17	4.29	0.00	0.00	0.00	33.42	33.84	33.78
121.00	4.21	4.27	4.23	0.00	0.00	0.00	32.10	32.45	32.32
145.00	4.38	4.59	4.49	0.00	0.00	0.00	31.10	33.66	32.13
169.50	4.72	4.84	4.77	0.00	0.00	0.00	32.22	33.19	31.34
193.00	4.62	4.87	4.67	0.00	0.00	0.00	31.33	33.13	32.46
220.00	4.79	4.86	4.87	0.00	0.00	0.00	31.47	31.78	33.13

Table 25: TP1 culture in 60% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.62	0.59	0.60	7.24	7.10	7.18	43.16	41.31	42.63
7.50	1.00	0.93	1.01	6.12	6.02	6.17	41.43	41.49	42.19
11.50	1.11	1.10	1.20	4.72	4.76	5.45	39.50	39.84	41.49
24.50	1.97	1.82	1.97	0.00	0.00	0.00	38.52	39.87	41.05
30.50	2.33	2.18	2.26	0.00	0.00	0.00	38.98	39.66	40.66
36.00	2.54	2.37	2.52	0.00	0.00	0.00	39.83	39.44	41.19
48.00	2.93	2.62	2.86	0.00	0.00	0.00	38.28	39.08	38.54
60.00	3.18	2.94	2.96	0.00	0.00	0.00	36.21	36.65	34.86
72.00	3.14	3.01	3.26	0.00	0.00	0.00	34.77	37.81	37.11
96.00	3.28	3.05	3.18	0.00	0.00	0.00	33.48	36.97	35.50
121.00	3.28	3.03	3.26	0.00	0.00	0.00	32.72	37.03	37.45
145.00	3.55	3.02	3.45	0.00	0.00	0.00	35.14	38.63	37.42
169.50	3.41	3.07	3.32	0.00	0.00	0.00	34.11	38.94	37.90
193.00	3.32	2.91	3.20	0.00	0.00	0.00	34.98	37.79	37.92
220.00	3.50	3.10	3.25	0.00	0.00	0.00	34.43	38.38	37.50

Table 26: TFA7 culture in 60% SSL media

	Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	0.77	0.77	0.82	7.20	7.16	7.21	42.52	42.94	41.56
7.50	1.22	1.27	1.38	6.13	5.92	4.88	43.22	41.10	41.27
11.50	1.52	1.65	1.84	6.45	5.15	4.85	43.13	40.63	40.42
24.50	2.77	2.78	3.07	0.00	0.00	0.00	39.31	38.07	39.35
30.50	3.04	3.16	3.45	0.00	0.00	0.00	39.25	38.76	38.61
36.00	3.05	3.45	3.56	0.00	0.00	0.00	39.06	39.14	37.76
48.00	3.90	3.58	3.95	0.00	0.00	0.00	37.69	33.29	33.58
60.00	3.95	4.06	4.26	0.00	0.00	0.00	34.71	34.14	33.98
72.00	4.28	4.48	4.47	0.00	0.00	0.00	35.00	34.72	33.52
96.00	4.56	4.87	4.64	0.00	0.00	0.00	32.82	33.21	29.97
121.00	5.01	5.11	5.30	0.00	0.00	0.00	33.93	32.21	31.14
145.00	5.15	5.58	5.85	0.00	0.00	0.00	32.66	32.43	31.78
169.50	5.41	5.58	5.96	0.00	0.00	0.00	32.60	32.01	30.42
193.00	5.21	5.81	6.03	0.00	0.00	0.00	33.42	32.11	30.59
220.00	5.30	5.65	5.99	0.00	0.00	0.00	32.45	31.56	30.76

Table 27: CelluX^{TM}4 two-pulse shake flask fed batch culture

		Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Volume (mL)	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	70.00	1.15	1.08	1.17	2.79	2.80	2.79	16.83	16.98	16.13
4.75	70.00	2.50	2.46	2.53	0.00	0.00	0.00	12.72	13.36	13.36
11.50	70.00	4.16	4.43	4.35	0.00	0.00	0.00	9.38	8.76	8.98
23.00	70.00	5.90	6.46	6.25	0.00	0.00	0.00	6.72	4.74	7.16
36.00	70.00	6.91	7.33	7.21	0.00	0.00	0.00	4.57	0.00	3.91
48.00	70.00	6.34	6.47	6.73	0.00	0.00	0.00	0.00	0.00	0.00
48.00	94.00	4.27	4.28	4.32	4.83	4.80	4.97	23.57	23.45	24.59
60.50	94.00	7.22	7.38	6.90	0.00	0.00	0.00	19.83	19.53	20.57
72.00	94.00	7.81	7.84	7.84	0.00	0.00	0.00	18.50	18.01	19.56
84.50	94.00	8.21	8.34	8.26	0.00	0.00	0.00	17.26	17.28	17.81
96.00	94.00	8.36	8.52	8.50	0.00	0.00	0.00	16.33	16.17	17.49
96.00	118.00	6.41	6.44	6.29	5.68	5.71	5.85	31.87	31.54	32.51
107.00	118.00	7.77	7.35	7.19	0.00	0.00	0.00	33.63	30.19	31.88
120.00	118.00	7.60	7.91	7.37	0.00	0.00	0.00	30.18	29.12	30.22
131.00	118.00	8.02	8.41	7.89	0.00	0.00	0.00	29.15	28.48	29.42
144.00	118.00	8.39	8.49	8.36	0.00	0.00	0.00	28.77	27.80	29.70
155.00	118.00	8.40	8.50	8.23	0.00	0.00	0.00	28.27	27.53	28.56
168.00	118.00	8.44	8.52	8.27	0.00	0.00	0.00	27.60	26.86	27.82
179.00	118.00	8.39	8.51	8.29	0.00	0.00	0.00	27.55	26.37	27.97
192.00	118.00	8.59	8.72	8.37	0.00	0.00	0.00	27.11	26.49	27.39
203.00	118.00	8.53	8.54	8.38	0.00	0.00	0.00	26.76	26.06	27.20
216.00	118.00	8.40	8.63	8.31	0.00	0.00	0.00	26.61	25.85	26.84

Table 28: CelluX^{TM}4 four-pulse shake flask fed batch culture

		Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Volume (mL)	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	70.00	1.13	1.16	1.09	2.76	2.76	2.36	15.67	16.75	11.77
4.75	70.00	2.50	2.56	2.02	0.00	0.00	0.00	12.35	12.92	9.22
11.50	70.00	4.78	4.80	4.63	0.00	0.00	0.00	8.89	8.65	6.33
23.00	70.00	6.32	6.32	5.67	0.00	0.00	0.00	6.92	6.12	3.37
36.00	70.00	6.94	7.19	5.96	0.00	0.00	0.00	3.55	3.16	0.00
48.00	70.00	6.34	6.56	8.90	0.00	0.00	0.00	0.00	0.00	18.27
48.00	82.00	5.10	4.85	4.30	3.43	3.46	2.85	15.54	15.66	12.99
60.50	82.00	7.60	7.63	6.58	0.00	0.00	0.00	11.11	12.22	8.99
72.00	82.00	8.20	8.40	7.47	0.00	0.00	0.00	9.99	10.51	6.90
72.00	94.00	6.82	6.90	6.37	4.14	4.23	3.59	20.82	21.50	16.75
84.50	94.00	8.23	8.41	7.78	0.00	0.00	0.00	17.57	19.06	14.32
96.00	94.00	8.68	8.79	8.33	0.00	0.00	0.00	16.87	18.17	13.09
96.00	106.00	7.40	7.27	7.33	4.56	4.67	0.00	26.42	27.22	22.05
107.00	106.00	8.26	8.19	8.05	0.00	0.00	0.00	24.90	25.56	20.31
120.00	106.00	8.72	8.78	8.44	0.00	0.00	0.00	25.83	24.72	19.57
120.00	118.00	7.60	7.47	7.54	0.00	0.00	0.00	36.88	31.95	27.02
131.00	118.00	7.97	7.85	8.19	0.00	0.00	0.00	30.72	31.28	25.82
144.00	118.00	8.36	8.19	8.40	0.00	0.00	0.00	29.62	30.20	24.73
155.00	118.00	8.50	8.56	8.64	0.00	0.00	0.00	29.30	30.00	24.27
168.00	118.00	8.68	8.44	8.77	0.00	0.00	0.00	28.52	29.06	24.59
179.00	118.00	8.60	8.31	8.74	0.00	0.00	0.00	28.14	28.73	23.85
192.00	118.00	8.74	8.60	8.69	0.00	0.00	0.00	27.97	28.53	23.56
203.00	118.00	8.87	8.67	8.63	0.00	0.00	0.00	28.57	28.44	23.16
216.00	118.00	8.69	8.57	8.62	0.00	0.00	0.00	27.75	29.02	22.68

Table 29: TFA7 two-pulse shake flask fed batch culture

		Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Volume (mL)	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	70.00	0.53	0.55	0.56	2.32	2.43	2.38	15.99	16.49	16.17
4.75	70.00	1.51	1.77	1.61	0.00	0.00	0.00	14.56	13.95	14.03
11.50	70.00	3.01	3.27	3.24	0.00	0.00	0.00	11.53	11.00	10.96
23.00	70.00	3.66	3.91	3.84	0.00	0.00	0.00	9.30	8.99	9.09
36.00	70.00	3.93	4.15	4.26	0.00	0.00	0.00	8.38	7.32	7.79
48.00	70.00	4.46	4.54	4.65	0.00	0.00	0.00	7.00	5.77	6.53
48.00	94.00	3.11	3.42	3.70	3.99	3.68	3.69	23.16	20.11	20.29
60.50	94.00	4.61	5.02	5.30	0.00	0.00	0.00	22.17	18.01	17.21
72.00	94.00	5.04	5.19	5.51	0.00	0.00	0.00	18.52	16.77	17.25
84.50	94.00	5.53	5.69	5.93	0.00	0.00	0.00	19.51	16.36	16.85
96.00	94.00	5.75	5.81	6.00	0.00	0.00	0.00	18.31	16.20	15.93
96.00	118.00	4.45	4.51	4.67	4.77	4.48	4.80	32.47	29.03	30.54
107.00	118.00	5.17	5.32	5.73	0.00	0.00	0.00	29.35	25.72	27.12
120.00	118.00	6.53	6.59	6.96	0.00	0.00	0.00	31.27	26.35	27.77
131.00	118.00	6.73	6.80	7.16	0.00	0.00	0.00	30.39	27.27	29.61
144.00	118.00	6.77	7.09	7.34	0.00	0.00	0.00	29.73	26.53	28.39
155.00	118.00	6.80	7.07	7.23	0.00	0.00	0.00	29.02	25.79	27.44
168.00	118.00	7.01	7.16	7.33	0.00	0.00	0.00	27.90	25.70	25.76
179.00	118.00	7.23	7.57	7.73	0.00	0.00	0.00	28.58	25.69	26.80
192.00	118.00	6.91	6.97	7.16	0.00	0.00	0.00	29.40	24.81	26.77
203.00	118.00	6.73	7.21	7.39	0.00	0.00	0.00	27.94	25.60	25.92
216.00	118.00	6.81	7.37	7.40	0.00	0.00	0.00	27.59	24.35	25.77

Table 30: TFA7 four-pulse shake flask fed batch culture

		Ethanol (g/L)			Glucose (g/L)			Xylose (g/L)		
Time	Volume (mL)	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
0.00	70.00	0.54	0.42	0.58	2.43	2.42	2.36	16.50	16.50	16.62
4.75	70.00	1.55	1.34	1.82	0.00	0.00	0.00	14.71	14.77	14.09
11.50	70.00	3.12	2.96	3.38	0.00	0.00	0.00	11.57	11.72	9.55
23.00	70.00	3.69	3.46	4.56	0.00	0.00	0.00	9.22	9.81	5.78
36.00	70.00	3.99	3.89	4.93	0.00	0.00	0.00	7.95	8.89	4.25
48.00	70.00	4.32	4.22	5.11	0.00	0.00	0.00	6.44	7.14	3.67
48.00	82.00	3.79	3.69	4.20	2.51	2.80	2.79	15.09	16.09	12.32
60.50	82.00	4.67	4.54	5.65	0.00	1.89	0.00	12.31	14.59	9.81
72.00	82.00	4.96	5.10	5.99	0.00	0.00	0.00	11.16	14.09	8.82
72.00	94.00	4.47	4.28	5.29	3.09	3.13	3.33	19.84	22.13	17.00
84.50	94.00	5.28	5.18	6.29	0.00	0.00	0.00	17.34	19.68	15.93
96.00	94.00	5.56	5.54	6.53	0.00	0.00	0.00	16.28	19.17	15.52
96.00	106.00	4.74	4.79	5.75	3.55	3.76	3.73	22.43	26.52	21.80
107.00	106.00	5.39	5.34	7.59	0.00	0.00	0.00	20.80	24.58	21.27
120.00	106.00	6.73	6.70	7.80	0.00	0.00	0.00	22.62	26.16	21.34
120.00	118.00	6.18	6.10	7.16	0.00	0.00	0.00	29.39	32.50	27.41
131.00	118.00	6.51	6.33	7.63	0.00	0.00	0.00	28.14	31.46	26.69
144.00	118.00	6.86	6.60	7.63	0.00	0.00	0.00	27.67	30.50	24.77
155.00	118.00	6.92	6.77	7.93	0.00	0.00	0.00	26.71	29.68	25.15
168.00	118.00	7.01	6.96	8.19	0.00	0.00	0.00	24.42	29.25	24.13
179.00	118.00	7.38	7.20	8.59	0.00	0.00	0.00	25.64	29.15	23.38
192.00	118.00	6.91	6.62	8.07	0.00	0.00	0.00	25.34	29.06	23.36
203.00	118.00	7.13	6.88	7.82	0.00	0.00	0.00	25.48	28.72	22.58
216.00	118.00	7.17	6.91	8.39	0.00	0.00	0.00	24.90	28.36	21.32

Table 31: Reactor 6-day fed batch culture

	Volume (L)		Ethanol (g/L)		Glucose (g/L)		Xylose (g/L)		Acetic acid (g/L)	
Time (h)	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
0.00	2.00	2.00	0.00	0.29	2.18	2.83	17.84	21.80	2.10	2.88
21.00	2.29	2.28	6.38	6.45	0.00	0.00	18.32	22.45	3.88	4.23
45.00	2.61	2.59	9.11	9.30	0.00	0.00	19.03	26.60	4.42	5.01
69.00	2.91	2.88	9.30	10.07	0.00	0.00	19.23	30.03	3.94	5.65
93.00	3.33	3.30	9.79	10.50	0.00	0.00	25.76	34.85	4.68	6.62
117.00	3.91	3.85	9.72	9.79	0.00	0.00	31.59	41.45	5.26	7.16
141.00	4.61	4.56	9.82	9.21	0.00	0.00	38.04	48.74	5.77	7.71
165.00	4.61	4.56	10.29	9.91	0.00	0.00	39.02	47.18	5.75	7.78
Pure SSL					12.75	14.70	88.72	99.69	9.53	12.69

Table 32: Reactor 9-day fed batch culture

	Volume (L)		Ethanol (g/L)		Glucose (g/L)		Xylose (g/L)		Acetic acid (g/L)	
Time (h)	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
0.00	2.00	2.00	0.29	0.23	3.04	2.84	27.19	21.31	2.89	2.67
21.00	2.17	2.18	6.85	6.98	0.00	0.00	23.33	15.35	3.90	3.64
45.00	2.39	2.41	9.06	10.16	0.00	0.00	25.53	17.62	4.25	4.33
69.00	2.59	2.61	9.25	11.73	0.00	0.00	29.64	19.65	4.30	4.98
93.00	2.98	2.98	9.89	11.95	0.00	0.00	35.93	23.82	5.45	5.59
117.00	3.30	3.33	10.54	12.43	0.00	0.00	42.54	30.09	6.06	6.32
141.00	3.64	3.70	9.73	12.52	0.00	0.00	43.00	33.98	6.51	6.77
165.00	3.99	4.04	9.94	12.39	0.00	0.00	50.16	39.20	6.96	7.04
213.00	4.68	4.74	9.96	11.64	0.00	0.00	57.70	43.57	7.40	7.44
Pure SSL					15.03	14.83	113.18	98.96	12.73	13.53

Table 33: Reactor 6-day fed batch double inoculum culture

	Volume (L)		Ethanol (g/L)		Glucose (g/L)		Xylose (g/L)		Acetic acid (g/L)	
Time (h)	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
0.00	2.00	2.00	3.53	3.76	2.77	2.83	22.96	23.24	2.83	3.01
21.00	2.31	2.27	11.60	11.41	0.00	0.00	16.19	16.76	3.99	3.86
45.00	2.61	2.58	13.19	13.94	0.00	0.00	21.53	20.54	4.73	4.90
69.00	2.93	2.90	14.84	15.09	0.00	0.00	26.14	24.86	5.62	5.58
93.00	3.38	3.32	15.29	15.44	0.00	0.00	32.07	30.41	6.57	6.47
117.00	3.95	3.88	14.88	15.43	0.00	0.00	38.64	36.98	7.36	7.38
141.00	4.65	4.58	14.07	14.55	0.00	0.00	43.43	42.86	7.96	8.01
165.00	4.65	4.58	15.43	16.39	0.00	0.00	-	-	-	-
231.00	4.65	4.58	17.02	17.04	0.00	0.00	-	-	-	-
Pure SSL					13.68	13.83	92.54	98.51	12.85	13.28

Table 34: Reactor batch culture

	Volume (L)		Ethanol (g/L)		Glucose (g/L)		Xylose (g/L)		Acetic acid (g/L)	
Time (h)	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2	Rep 1	Rep2
0.00	4.60	4.60	0.00	0.00	9.79	10.50	66.11	66.80	8.15	8.16
69.00	4.60	4.60	1.89	0.00	5.19	9.67	62.66	68.06	7.64	8.07
93.00	4.60	4.60	2.77	0.00	0.00	9.95	62.54	66.56	7.69	7.94
117.00	4.60	4.60	3.30	0.00	0.00	9.84	62.46	66.51	7.67	7.79
141.00	4.60	4.60	3.28	0.00	0.00	9.51	61.13	67.06	7.56	7.95
165.00	4.60	4.60	3.29	0.00	0.00	9.18	61.06	65.40	7.69	7.86